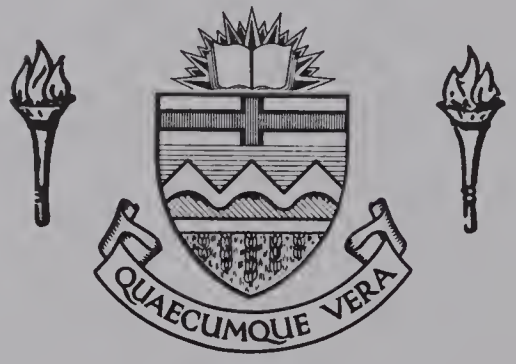

THE UNIVERSITY OF ALBERTA

BIOCHEMISTRY OF THE ANTERIOR SEGMENT OF THE EYE - THE ENZYMES OF THE CORNEA AND THE AQUEOUS -

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled BIOCHEMISTRY OF THE ANTERIOR SEGMENT OF THE EYE - THE ENZYMES OF THE CORNEA AND THE AQUEOUS submitted by JOO OK KIM, M.D. in partial fulfilment of the requirements for the degree of Master of Science (Ophthalmology).

ABSTRACT

Studies of several enzymes and substrates related to glucose metabolism have been carried out in normal corneas, and aqueous of human, and cat eyes.

In summary:

1. Studies on the "corneal temperature reversal effect" re-emphasize the fact that the "effect" is a metabolic function of the cornea, and suggest that glucose in the aqueous plays a major role.

   A secondary role is probably played by a substrate-reservoir in the cornea related to the degree of aerobic metabolism.

   The role of a physico-chemical influence in producing the temperature reversal effect was ruled out by almost complete loss of the effect in eyes stored at room temperature for two days.

2. A method for assessing corneal endothelial viability in eye-bank eyes without damaging the cornea is suggested. This involves analysis of aqueous for endothelial-cell specific enzymes, especially LDH and its isozymes.

   Based on statistical studies using the aqueous of human eyes, it has been concluded that below 50 mU/ml. of LDH activity in the aqueous can be

used as a borderline value to select corneas from an unknown environment.

3. LDH isozyme patterns of the corneal endothelium have been investigated in the human and cat. The former showed a "Muscle" type and the latter showed a "Heart" type of isozyme pattern.

The kinetics of LDH inactivation in vitro upon exposure to cold have been investigated in corneal endothelial extracts of human and cat. A much higher inactivation rate constant has been found with the former than with the latter.

After a discussion the features of corneal endothelial metabolism under standard eye-bank conditions, an attempt has been made to explain the species difference in vulnerability of endothelial cells to damage during storage of the enucleated eye at 4°C, utilizing the findings reported here.

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I. INTRODUCTION

Since Warburg (118) observed that the enzymes involved in tissue metabolism appear in serum, serum enzymes have been studied as a means of learning about cellular metabolism and changes in it.

It is presumed that enzymes of the corneal endothelium will pass into the aqueous humor as a result of increased cell membrane permeability, when the energy available to the cell is no longer sufficient to maintain the cell wall.

A high rate of aerobic glycolysis (64) has been reported in the cornea, and Kinoshita et al (56) have estimated that in bovine corneal epithelium, about 65% of the glucose is metabolized by the conventional glycolytic pathway and 35% by the shunt mechanism. Enzymes of the citric acid cycle of Krebs are present in the epithelial and endothelial layers of the cornea (1,60).

The present research originally aimed to develop a method for assessing corneal viability in eye-bank eyes without damaging the cornea for future use.

Three enzymes participating in glucose metabolism have been studied; lactic dehydrogenase (LDH) for the conventional glycolytic pathway, glucose-6-phosphate dehydrogenase (G-6-PDH) for the shunt mechanism, and isocitric dehydrogenase (ICDH) for the citric acid cycle. It was

found that of the three, the LDH level in the aqueous was most significantly changed with increasing storage of the whole eye at 4°C.

LDH was, therefore, analyzed in the aqueous and its levels related to quantitative damage to the endothelium; LDH isozymes were characterized in the aqueous after damage to the endothelium as well as in the tissue extract of the endothelium. The release of LDH from endothelial cells was also established by the concomitant decrease in enzyme activity in the endothelium.

The levels of LDH were then related to the temperature reversal effect (TRE). This effect has been described by many authors and is assumed to be dependent upon a metabolically active cornea (14,13,33,34,36,67). Many other experiments (4,17,32,51,66,62,81) have shown that the TRE is produced mainly by endothelial activity although the epithelium and to a lesser extent the stroma are involved. This effect has been used as a measure of endothelial viability in attempting to assess corneas as donor material, however, it has been shown to persist for 6 days storage in pig eyes (68), and cat eyes (37), long after a human cornea would be discarded as donor material by other criteria. Furthermore, Itoi (45) demonstrated a TRE with a hydrophilic soft contact lens, suggesting that it is a physicochemical effect of polyelectrolyte gels.

On the other hand, Reim and Turss (98) using bovine eyes analyzed some metabolite levels in the endothelium and aqueous, and suggest that the endothelium has functional activity until 30 hours of storage in a moist chamber at room temperature.

During the preliminary study on enzymes in the aqueous humor, a reverse TRE was observed on corneas of cat eyes of short term storage in which the aqueous had been removed and replaced by normal saline. This chance finding has led to studying the nature of TRE by analyzing several metabolites in the aqueous.

It is interesting that, while the endothelial cells of corneas of the cat (37), pig (70), and rabbit (102) are still metabolically active at a prolonged storage period, penetrating homografts using donor corneas after 24 hours storage are rarely successful in humans.

Direct studies have been made of the LDH isozyme pattern of human and cat corneal endothelium to determine the pattern of corneal endothelial LDH isozymes in the different species. This work has been undertaken in the hope that a variation in LDH isozymic pattern of the corneal endothelium would be found which could shed some light on the species difference in the vulnerability of endothelial cells to damage when stored at 4°C.

Early attempts to fractionate the LDH of the

cornea were made by Moore and Wortman (84) and Futterman and Kinoshita (25), both suggesting only three isozymes, but Graymore and McCormick (28) have shown that both in stroma and in the epithelium of the rabbit all five isozymes can be demonstrated. According to this report, the isozyme pattern in the epithelial-endothelial (E-E) fraction is of "Heart" (H-) type, which is characteristic of tissues with a high oxygenation (6), while the stroma shows the "Muscle" (M-) type of distribution indicating a more anaerobic metabolism (6). Five isozymes have also been found in whole corneal extracts of the ox and rabbit by Bernstein et al (3). They found isozymes 3-5 predominating in the adult bovine cornea, but isozymes 1-4 in the rabbit cornea. These authors did not separate stroma and epithelium before analysis.

Maurice and Riley (76) sought a possible explanation of this species difference based on a difference in the LDH ratio between the corneal layers in the two species. In the rabbit the 100-fold higher concentration of LDH in the epithelium could result in the isozyme pattern of this layer obscuring that of the stroma. In the ox, where the LDH ratio between the layers is only about 40, a composite pattern could be seen.

It is still an experimental fact, however, that intact epithelium of the bovine cornea does not exhibit aerobic glycolysis (101), a finding which is in contrast to

findings on the rabbit epithelium (64). Also, the epithelium of the bovine cornea is rich in lactic acid content (38).

Hence, in the course of the investigation, the purpose of the present work can be finalized in the form of the following questions:

1. What is the nature of TRE?

2. Is there any enzyme in the aqueous, which provides a practical tool for assessing corneal endothelial viability?
   If so, what is the normal level of the enzyme and what is the borderline value for the selection of donor corneas?

3. Why is there species difference in the vulnerability of corneal endothelial cells to damage when stored at 4°C.? Is there any difference in "Metabolic Control Mechanism"?

II. THEORY

A. FUNCTION AND METABOLISM OF THE CORNEA

A little over a decade ago, the demonstration of the TRE suggested that hydration of the cornea was metabolically controlled (13,14,33,34,36,65,67). It was known that the cornea would swell when eyes were maintained in a moist chamber at refrigerator temperatures and it was demonstrated that upon returning the intact chilled eye to body temperature the cornea dehydrated to its normal level.

Since energy is required to dehydrate a swollen cornea against its swelling pressure, an active mechanism concerned in removing the fluid is indicated. Furthermore, since the permeability data suggest that aqueous humor must be continuously drawn into the cornea of the intact eye from the anterior chamber, some active means of removing it under normal circumstances must be envisaged (73). Considerable indirect evidence points to this active mechanism being located in the endothelial layer (4,17,32,51,66,62, 81,113).

The deprivation of oxygen from the enucleated eye (13,36), or from the endothelial surface in particular of the excised cornea (114), shows the direct metabolic involvement of the tissue in the transport mechanism. The importance of the metabolic activity in the secretion of ions or fluid is re-emphasized by Harris (35) by determining Q_{10}.

The temperature coefficient, Q_{10}, is the ratio of the rate of a reaction at a given temperature to the rate of the same reaction occurring at a temperature 10°C lower:

$$Q_{10} = \frac{K_t + 10}{K_t}$$

Most physical processes show a low Q_{10}, the Q_{10} for simple diffusion, for example, of both electrolytes and nonelectrolytes is less than 1.5 and the ratio for many chemical reactions is 2. Reactions peculiar to biological systems, on the other hand, show a much higher Q_{10}. According to Harris' experiment, the Q_{10} of fluid excreted from the previously refrigerated cornea is 5. This indicates that the fluid excretion is operated through a pump mechanism located in the endothelial layer.

In spite of a number of experimental studies, the relationship between metabolism and the pump mechanism has remained obscure until recently (78). This is largely because the majority of early investigations were carried out on the intact eye-ball where little control can be exercised over the milieu of the endothelial layer. Donn et al (19) were first able to assess the activity of the pump from measurements of the corneal thickness, by mounting the isolated cornea between chambers so that fluids could be passed over the surfaces separately, with a

hydrostatic pressure between them corresponding to the normal intraocular pressure. In this manner, Mishima and Kudo (81) tested a number of incubation media for their efficacy as aqueous humor substrates. The KEI medium, a medium developed as an aqueous substitute in lens incubation studies by Wachtl and Kinsey (117) was found to be the best of all solutions tested.

This solution allowed the maintenance of normal corneal thickness for over 10 hours and nearly complete temperature reversal of the cold-stored swollen cornea. This medium is a balanced salt solution gassed with 7% O_2, 88% N_2 and 5% CO_2 which contains amino acids and a large range of metabolic intermediates in addition to glucose and pyruvate. If the solution was made anoxic, Takahashi (114) showed that irreversible corneal swelling took place even if 95% O_2 was supplied to the epithelial surface.

It was found that as long as 1 mM of reduced glutathione was present in a balanced salt solution the only substrate required was glucose (78). It was also found (78) that the glucose could be replaced by fructose or adenosine, whereas metabolites beyond the level of energy-rich intermediates of the glycolytic chain were not adequate substrates, e.g. pyruvate, α-keto-glutarate and succinate. The authors suggested, in the light of this experiment and the fact of glutathione stabilizing the action of 3-phosphoglyceralde-

hyde dehydrogenase, that the action of this enzyme or one of the next stages of glycolysis is essential to the pump mechanism.

They also suggested that a synthetic process would be involved, since the pump was inhibited by antibiotics such as puromycin (20 μg ml^{-1}) and actinomycin (0.1 μg ml^{-1}) which are inhibitors of protein synthesis. Puromycin is known to inhibit protein synthesis at assembly stage on ribosome and actinomycin inactivates DNA specifically needed for RNA synthesis.

They have then concluded that two steps which operate at the same time are responsible for the endothelial transport mechanism: One involves a synthetic process and the other is a stage in the glycolytic pathway, probably that controlled by 3-phosphoglyceraldehyde dehydrogenase and phosphoglycerokinase.

A clouding of the isolated ox cornea was observed with storage in CO_2 which was reversed if the cornea was returned to air within the first few hours (96). In the epithelium of this ox cornea, a steep fall in the ATP/ADP ratio was found from a value of 17 to 4 in the first 30 min. The levels of G-6-P and FDP also showed rapid changes, rising to double their initial value and returning again within 5-10 hours. The metabolite levels also partially recovered their original values if the tissue was returned to air.

Earlier studies on the whole rabbit cornea by Reim and Lichte (95) also showed a close relation between ATP/ADP-quotient and turbidity of the isolated cornea. It was not definitely shown, however, that these changes in transparency occurred other than in the epithelial layer itself, but this would appear to be a likely hypothesis in the absence of the possibility of swelling or structural disorganization of the stroma.

B. PATHWAYS OF GLUCOSE METABOLISM AND HYDROGEN TRANSFER IN THE CORNEA

The early studies on glucose metabolism as the chief source of energy for the cornea are conflicting, and as de Roetth (101) has pointed out, tend to confuse rather than clarify the overall picture. The work of de Roetth, however, coupled with reports from Hermann and Hickmann (38), Langham (63,64), and a vast and informative literature from Kinoshita et al (53) enable one to visualize a general picture characterized by a very high degree of metabolic activity in the epithelial and endothelial layers of the cornea as evidenced by an oxygen consumption of approximately 6 µl per mg per hour (64), high levels of enzymes (1, 60) and of pyridine nucleotides (85). However, the main bulk of the cornea, the stroma, which consists largely of collagen imbedded in a polysaccharide ground substance, is sparsely populated with cells and appears to be relatively inert.

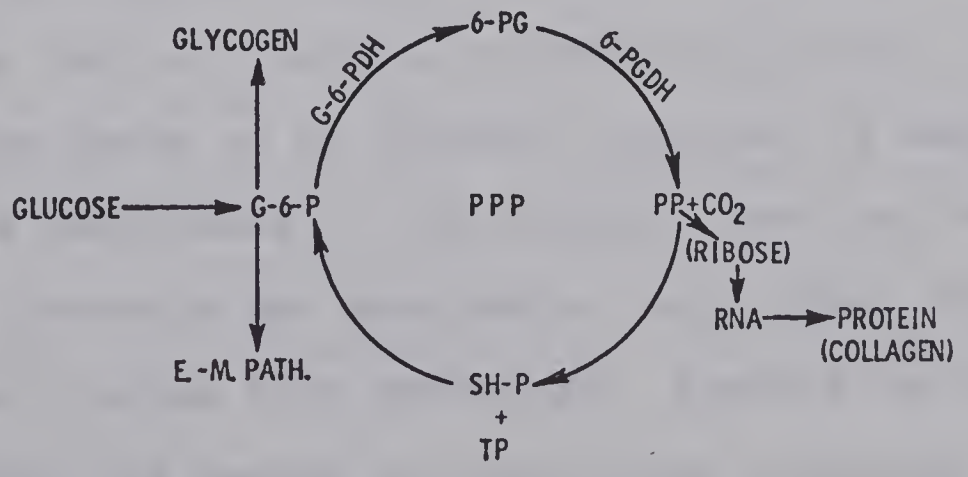
Interaction between layers was suggested by Hermann and Hickmann who found that in an excised, intact cornea the lactate depot in the stroma was depleted upon incubation. In contrast, the disappearance of lactate from the stroma of a denuded cornea was not observed. The possibility that the lactate formed by the stroma could be utilized by the epithelium has also been considered by Graymore et al (28) on the basis of the difference in LDH isozymic pattern between the epithelial-endothelial fraction and the stroma.

However, it would appear, from work of Hermann and Lebeau (40), which has been carefully controlled, that the three layers of the cornea are largely self-sufficient with regard to their glucose metabolism and energy supplies. An epithelial barrier function was demonstrated by these authors who showed that scraping off the epithelium with a scalpel led to a loss of 87% of the ATP in the stroma, and caused a drastic reduction in the incorporation of glycine into the collagen of this tissue. However, if the epithelium was removed more carefully with a brush, while the eye was still in situ, the loss of ATP was reduced to 48%, and no disturbance of the tracer glycine into the stroma proteins was observed. Similar conclusions (79) have been reached in regard to the importance of the epithelium in the incorporation of sulphate into the polysaccharide of the stroma.

1. Epithelium

Kinoshita et al (56) have estimated that in bovine corneal epithelium about 65% of the glucose is metabolized by the conventional glycolytic pathway and 35% by way of the shunt mechanism. The importance of the shunt pathway was confirmed by Kuhlman and Resnik (61) who studied the ^{14}C-labelled glucose and lactate oxidation of whole cornea. Both substrates have been shown to be oxidized to $^{14}CO_2$, also convincing evidence for the active citric acid cycle.

The functioning of oxidative phosphorylation is revealed by the high ATP/ADP ratio and its rapid decline under anaerobic conditions (95) as well as by stimulation of respiration by low concentrations of dinitrophenol (67). The pathway of glucose oxidized by the shunt mechanism involves the two-step oxidation of glucose-6-phosphate to yield ribulose-5-phosphate (54,56) and Kinoshita (53) showed a complete cycle of the pentose phosphate by trans-aldolase and transketolase to yield glucose and triose phosphate.



Relationship of Pentose Phosphate Pathway to Protein Synthesis

In the preceeding cycle, there are two dehydrogenase reactions which require nicotinamide-adenine dinucleotide phosphate (NADP) in the oxidized form. In these reactions NADP is reduced to (NADPH) by accepting electrons from the substrates oxidized. Since the coenzyme is available only in limited quantities, once reduced it must be reoxidized.

The mode of reoxidation of NADPH produced in the above cycle has not been conclusively elucidated in the cornea (77), but Kinoshita (53) has suggested that pyruvate functions as an electron acceptor for NADPH in the cornea. He has shown the oxidation of NADPH through the LDH reaction and that glucose oxidation through the shunt mechanism are inhibited by low levels of iodoacetate, which is known to inhibit triose phosphate dehydrogenase. The iodoacetate inhibition of the shunt mechanism is overcome by the addition of pyruvate. He has summarized the known facts concerning the reoxidation of NADPH by pyruvate as follows (53): The NADPH formed in the dehydrogenation and decarboxylation of glucose phosphate through the shunt mechanism can be reoxidized by the LDH reaction converting pyruvate to lactate. Pyruvate can serve as an electron acceptor of NADPH provided that NAD is maintained in the oxidized form and sufficient amounts of pyruvate are available. A pH lower than 7.4 greatly facilitates this mechanism. Lactate can be regarded as a <u>reservoir of energy</u> in that it can eventually cause a

reduction of NAD which, in turn, can be channeled into the electron transmitting system for the production of ATP. In effect, the NADPH formed in the shunt mechanism is converted to NADH. Thus, the coupling of the dehydrogenases of the shunt mechanism with LDH provides the tissue with a trans-hydrogenation mechanism of the coenzyme.

Kuhlman and Resnik (61) have criticized the hypothesis of this trans-hydrogenation system via LDH in the regeneration of NADP on the grounds that the pyruvate formed from the Embden-Meyerhof cycle would be oxidized along with that derived from the oxidation of lactate via the citric acid cycle; they propose that NADPH is oxidized through the cytochrome system.

On the other hand, quantitative evaluation (77), from the available data, of the pathways of glucose utilization in the epithelium suggests that all the lactate produced in this layer could arise from reaction of pyruvate with NADPH from the G-6-PDH and 6-PGDH reactions. In the case of Kinoshita's suggestions concerning the role of pyruvate, it would be required for an efficient shuttle path, such as the α-glycerophosphate $\longleftrightarrow$ dihydroxyacetone phosphate or β-hydroxybutyrate $\longleftrightarrow$ acetoacetate systems, to transfer reducing equivalents of the NADH which is also produced into the mitochondria for oxidation by the phosphorylating system.

There are other possible schemes of the NADPH-linked dehydrogenases by which reoxidation may take place: First, it is now generally accepted that in most tissues NADPH formed in the shunt mechanism serves as a hydrogen donor in the synthesis of fatty acid and steroids; second, in the cornea, there are two other dehydrogenases which might participate in similar reactions. One of them is glutathione reductase and the other isocitric dehydrogenase. Carson (7) has pointed out that in erythrocytes the interaction of the dehydrogenases of the shunt mechanism and glutathione reductase controls the levels of glutathione. A defect in the G-6-PDH, as observed in the primaquine-sensitive individuals, produces a lowering of the glutathione content in red cells. This interaction of dehydrogenases is also demonstrable in the corneal epithelium (54) and in the lens (55), where glutathione is in abundance. But there is no efficient system known for regeneration of the oxidized glutathione in the cornea.

The enzyme, ICDH, can utilize both NAD and NADP as coenzymes, but reacts $2x10^4$ faster with the latter nucleotide (15). A high content of ICDH is demonstrated in bovine epithelial cells, while in rabbit, LDH is ten fold more concentrated (60,97,116).

2. Stroma

Langham (64) calculated that in the rabbit cornea

the rates of respiration and lactate production are about twenty-seven times greater in the epithelium than in the stroma, on a weight basis. As pointed out by Hermann and Hickmann (38), the apparent metabolic inactivity of the stroma is due to the abundance of the connective tissue component. If the rate of metabolism were expressed on a basis of cellular content, the stroma would probably prove to be as active as the epithelial layers.

Histochemical data (1,60) and quantitative enzyme assays (15,97) suggested that the stromal cells appear to be unable to oxidize glucose via the pentose phosphate pathway. Like Baum (1), Reim and Schramm (97) were unable to measure 6-PGDH in the rabbit stroma, but Delbruck (15) did find a very low activity in the ox stroma. He was, however, unable to detect G-6-PDH activity in this layer and therefore the complete enzyme complement for glucose oxidation by the pentose phosphate pathway appears to be absent from the stroma of this species. It is of interest to note, however, that Buñuel and Buñuel have observed that the activity of G-6-PDH and 6-PGDH is increased in cells producing new connective tissue, possibly in response to the increased requirement for ribose in RNA synthesis prior to collagen formation. It seems possible that these enzymes would be detectable in keratocytes of developing or regenerating corneas as in wound repair.

3. Endothelium

The endothelium appears to possess all the enzymes that have been demonstrated in the epithelium. The total cell volume of this layer in different species is between 5 and 10% of that of the epithelium, and Hermann and Hickman (39) measured the rate of respiration in the isolated endothelium to be 7% of the epithelial rate in the ox. This suggests that the rate per cell is about equal in the two layers, a conclusion which has been reached by others (64,100) who calculated the activity from the difference in oxygen consumption rates of the stroma with and without endothelium.

The endothelial cells have a much greater number of mitochondria than those of the epithelium or stroma and might, therefore, be expected to exhibit a greater oxidative rate. However, the cristae are aligned parallel to the axis of the mitochondrion (46), a formation which indicates a low oxidative capacity according to Fawcett (22), in contrast to the more usual transverse cristal arrangement found in highly active mitochondria. As discussed in the section on function and metabolism of the cornea, the energy requirement for dehydration is small. Apart from this energy requirement of uncertain magnitude connected with the dehydration mechanism, the only synthetic activity evidently associated with the endothelium is the very slow thickening

of Descemet's membrane. The function of the pentose phosphate pathway of glucose oxidation is reported to occur in this layer (61).

C. REACTIONS TAKING PLACE IN THE AQUEOUS

1. Ascorbate and Glutathione

In the previous section, it has been pointed out that reduced glutathione is essential to the endothelial pump mechanism. However, there is no glutathione present in the aqueous humor of the bovine eye (41). It has been reported to be present in the ox cornea in the amount of approximately 0.8 micromoles g^{-1} wet weight and entirely in its reduced state.

It has been found also in the lens cortex, lens nucleus, retina, ciliary body and iris. It is present at a much higher level in the ox epithelium than in the stroma. Expressed in terms of glutathione concentration per cell, however, the levels in the epithelial cells and the stromal cells are very similar.

The aqueous humor in certain species, on the other hand, has a high ascorbic acid content, actively transported from plasma.

According to Pirie (91), ascorbic acid is autoxidizable at the pH of the aqueous with the formation of traces of peroxide:

$$\text{Ascorbate} + O_2 \longrightarrow \text{Dehydroascorbate} + H_2O_2$$

Pirie (91) has identified both dehydroascorbate and peroxide in cattle eyes. The reaction is catalyzed by traces of riboflavin and either the dehydroascorbate or the peroxide serves to oxidize the glutathione:

$$2\ GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{Glutathione}} GSSG + 2\ H_2O$$

$$\text{Dehydroascorbate} + 2\ GSH \xrightarrow[\text{Non-enzymatic}]{} \text{Ascorbate} + GSSG$$

Oxidized glutathione can be re-reduced by NADPH and glutathione reductase:

$$GSSG + NADPH \xrightarrow[\text{reductase}]{\text{Glutathione}} 2\ GSH + NADP^+$$

Kinoshita and Masurat (55) found that the supply of NADP in the lens limits the oxidation of glucose by the pentose phosphate pathway and that, in certain circumstances, addition of oxidized glutathione stimulates oxidation of glucose.

Pirie (91) has suggested, therefore, that the whole series of reactions, starting with uptake of oxygen by ascorbic acid in the aqueous may be required in lens metabolism.

Kinoshita and Masurat (55) also measured gluta-

thione reductase in the cornea and found a fairly constant ratio between the level of this enzyme and the levels of G-6-PDH and 6-PGDH. Therefore, if glutathione plays any role in the regeneration of NADP for the pentose phosphate shunt in the cornea, the auto-oxidation of ascorbate in the aqueous may be required for regeneration of the oxidized glutathione.

It is not known where the equilibrium of this reaction lies but the catalysis by riboflavin appears to be light-sensitive (91).

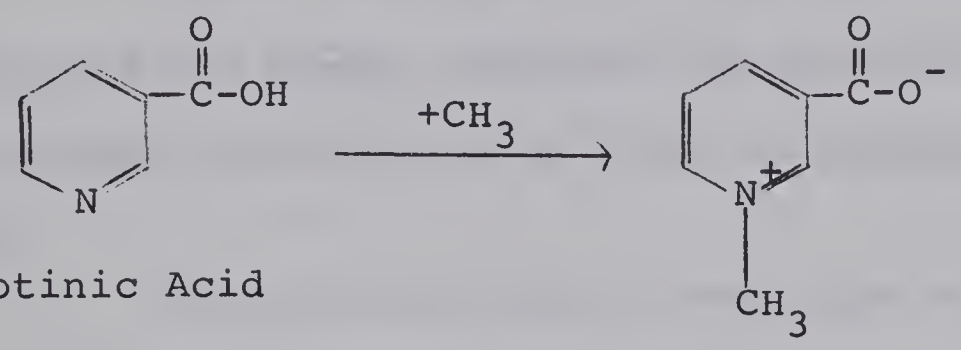
2. Transmethylation Reactions

A source of labile methyl is an important factor in fat metabolism since substances which supply methyl groups act indirectly as lipotropic agents by contributing to the synthesis of choline. Methionine is the most important source of labile methyl and betaine, an oxidation product of choline, is another methyl donor, although formation of methionine is the actual route by which betaine acts as a methyl donor (31).

Transmethylation also occurs in the metabolic processes of nicotinic acid: The largest portion of niacin derivatives is excreted as methyl derivatives.

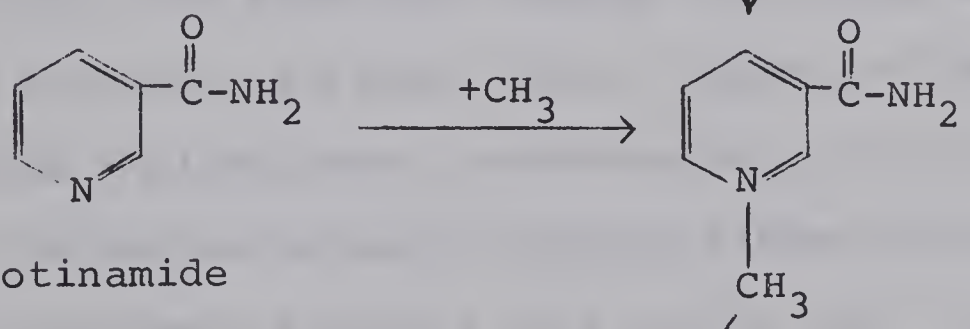
Transmethylation and formation of nicotinic acid and its derivatives from tryptophan occur regularly in the aqueous humor and in the lenses of normal human and ox eyes.

The transmethylating enzymes present in the aqueous probably occur due to diffusion of enzymes from the lens (9); in eyes with cataract, transmethylation does not occur and nicotinic acid derivatives are not formed from tryptophan in the lenses or in the aqueous; although no free tryptophan is present in the aqueous humor of eyes with cataract, it does occur in the cataractous lens.



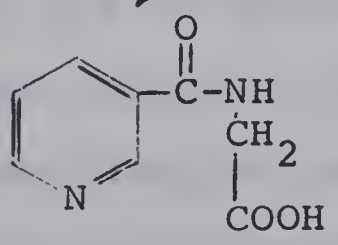
Nicotinic Acid

Trigonelline



Nicotinamide

Trigonellinamide



Nicotinuric Acid

3. Thromboplastin in Reactions

Normal aqueous does not contain fibrinogen. When the blood-aqueous barrier becomes abnormally permeable, however, in this plasmoid aqueous thromboplastin reactions can take place (11).

D. EXCHANGES BETWEEN AQUEOUS AND CORNEA

Since the cornea is avascular, it must derive the materials and oxygen required for metabolism either from the pericorneal capillaries or from the aqueous humor and tears (86).

The availability of materials which enter from the limbus will depend (i) on the rate of diffusion of the substance through the corneal stroma, and (ii) on the rate at which the substance leaves the stroma across the anterior and posterior surfaces (12). Kaiser and Maurice (49) showed a significant concentration of protein in the center of the cornea because although plasma protein entering from the periphery diffuses very slowly into the stroma, the corneal endothelium and epithelium greatly restrict its loss from the stroma. In the case of small molecules, the rate of loss from the anterior and posterior surfaces is relatively greater and it appears that as far as the principal metabolites are concerned, the vascular system directly supplies only a very limited peripheral region of the cornea (74) and the nutrition of the central regions must therefore

be derived to a great extent from the aqueous humor.

1. Glucose

There are conflicting opinions regarding the transport of glucose out of the aqueous humor across the corneal endothelium.

Cole (12) believed that glucose is actively transported into the interstitial fluid of the corneal stroma. This theory is based upon the finding of Graymore (26), that there was no difference between the glucose levels of the aqueous humor and of the corneal stroma of rabbits.

On the other hand, Reim et al (94) studied the level of glucose in blood plasma, aqueous humor, tears and different layers of the cornea with respect to the concentration gradients in different species. They found that the glucose concentration in the aqueous humor was almost twice that in the corneal endothelium and extracellular fluid of the stroma.

An experiment with bovine eyes stored in a moist chamber at room temperature revealed that glucose levels in both the aqueous humor and in the endothelium fell in parallel to almost zero within ten hours. At this time the cornea was completely clear and the adenosinphosphate levels and the ATP/ADP ratios in the endothelium were almost normal (98). From these results, they concluded that the

barrier between the anterior chamber and the corneal stroma is in the endothelium and that the endothelial glucose level varies in relation to the level in the aqueous humor which is passively diffused into the endothelium following a concentration gradient.

2. Lactate

Langham (64) suggested that unmetabolized lactate leaves the cornea by passive diffusion across the endothelium into the anterior chamber aqueous. This suggestion is based on his finding that the lactate concentration is 9.4 micromoles g^{-1} in the cornea as opposed to 7.3 micromoles ml^{-1} in the aqueous of the rabbit. Reim and Turss (98) also reported a similar concentration gradient of lactate between the corneal endothelium and the aqueous in bovine corneas.

3. Oxygen, Carbon Dioxide

Fatt and Bieber (20) have calculated the flux of gas across the surfaces of human cornea and the changes in tension across its thickness. According to this report, under normal aerobic conditions in the open eye there is a fairly steady fall in O_2 tension from 155 mmHg in the tear film to 55 mmHg in the aqueous humor. These authors found the O_2 tension of the palpebral conjunctiva also to be 55 mmHg. In the closed eye there is an entry of gas from both

surfaces and a minimum tension near the middle of the stroma.

If the eye is covered, as with a contact lens, or is placed in a N_2 atmosphere, the epithelium will be in respiratory distress. Under these conditions the endothelium can most probably manage adequately with O_2 provided by the aqueous humor (114).

Fatt and Bieber (20) have also calculated CO_2 distribution across the cornea on the same basis as for O_2. Owing to the far greater solubility of this gas it is transferred at a much greater rate and the tension profiles are mainly straight lines when the epithelial, stromal and endothelial levels are compared. In the open eye the aqueous should release CO_2 through the cornea into the virtually CO_2-free air. The efflux from the human cornea in vivo was measured by Fatt et al (21) by a CO_2 electrode in a contact lens and was found to be 21 µl per hour for the whole corneal surface. However, the values calculated theoretically by Fatt and Bieber are almost double these. In vivo, therefore, most of the corneal CO_2 which is produced by the epithelial layers, is lost directly across this surface to the precorneal tear film and a much smaller proportion which is formed in the endothelium, diffuses into the aqueous humor.

4. Water

The outer surface of the cornea is covered by a

film consisting, according to Mishima (80), of three layers: Mucopolysaccharide, lacrimal fluid and an oily film derived from Meibomian secretion. Mishima and Maurice (82) have investigated the evaporation from the fluid layer and found that about 3 µl $hr^{-1}cm^{-2}$ of evaporation occurs from the normal tear surface under standard indoor conditions.

The tears are approximately isotonic with aqueous (83,58,72,89,103) the tonicity being dependent upon the dynamic interrelationship of the rate of tear secretion, tear volume, rate of evaporation, and rate of water movement from the aqueous. The rate of loss of aqueous by this route depends, therefore, on the rate of evaporation from the lacrimal fluid, and the layer of Meibomian secretion, which may well be only a few molecules thick, reduces the rate of evaporation very considerably. In the absence of this layer, enough water leaves the anterior chamber to cause a fall of intraocular pressure.

It will be noted (12) that the transcorneal movement of water constitutes, in fact, one step in an exchange between the aqueous humor and the external environment with the precorneal tear film acting as a protective mechanism to reduce the net loss of water. Nevertheless, the dehydration of the cornea by evaporation is a factor in the maintenance of corneal hydration (35) although the epithelium does not appear to be involved in the excretion of fluid

from the cornea.

Whilst this dehydrating system is probably effective in most terrestrial mammals the problem of maintaining both a correct fluid balance and normal corneal hydration must be quite different in a semi-aquatic and aquatic species. Various adaptations and evolutions to special conditions are met in these species (12).

The dehydrating system in relation to the metabolic pump mechanism of the endothelium has been described in the section on "Function and Metabolism of the Cornea".

III. EXPERIMENTAL

A. RELATION OF TRE TO STORAGE TIME AND TO AQUEOUS GLUCOSE AND METABOLITE LEVELS

1. Introduction

This experiment has been designed to assess the TRE in relation to the level of metabolites and the main nutrient, glucose, in the aqueous. Both eyes of an adult cat were used as an experimental pair. After enucleation and cooling to 4°C in a moist chamber, the aqueous of one eye "A" was removed and replaced by normal saline. Analysis was carried out on the aqueous for glucose, lactate and pyruvate. This eye, with saline in the anterior chamber, and the second eye "B" with aqueous in the anterior chamber were then incubated at 37°C for two hours in Eagle's media with 95% O_2 and CO_2. After incubation the second eye "B" was also analyzed for lactate and pyruvate in the aqueous.

2. Methods and Materials

(a) Enucleation of Eyes and Collection of Aqueous Humor: Cat eyes were enucleated under semi-sterile conditions from a living choline anaesthetized adult cat, and placed in a moist atmosphere of normal saline. The aqueous humor was collected by anterior chamber puncture using a 1.0 ml syringe and a 25 G needle inserted at the corneal limbus.

(b) TRE Measurement and Calculation

(i) Pachymetry: The corneal thickness was measured according to the procedure described by Maurice and Giardini (75).

(ii) TRE Calculation: The TRE was calculated as the percentage reversal of the increase in thickness produced by storage at 4°C.

$$\text{Temperature Reversal (TR)} = \frac{\text{Change in thickness with incubation}}{\text{Increase in thickness with storage}} \times 100$$

The TR was noted as being negative (-) if corneas showed an increase in thickness on incubation instead of a reduction in thickness. This result occurred only in the eyes incubated without aqueous. The temperature reversal for fresh eyes was attempted by incubating the eye for two hours, and measuring corneal thickness before and after incubation. Because there is no cooling, theoretically fresh corneal thickness is the same as thickness after storage. Experimentally the corneal thickness after incubation was the same as that in the fresh state. Thus, fresh eyes showed a complete TR.

Four cat eyes stored at room temperature (+23°C) in a moist chamber were also subjected to a calculation of the TRE.

(c) Determination of Glucose: Glucose was measured by means of the hexokinase reaction, described by Slein (105)*. Glucose in the aqueous reacts in the presence of hexokinase and Mg^{++} with ATP to produce glucose-6-phosphate (G-6-P). The reaction of G-6-PDH is used as the indicator system in which the absorbancy of the produced Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) at 340, 334 or 366 nm can be measured with the Beckman DB-G Spectrophotometer.

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^{+} \underset{}{\overset{\text{G-6-PDH}}{\rightleftharpoons}} \text{6-PG} + \text{NADPH}$$

(6-phosphogluconate)

In a total volume of 3.00 ml, 0.2 ml consisted of undiluted or x10 diluted aqueous humor, 2.60 ml of 0.3 M triethanolamine buffer, pH 7.5 with 4 mM $MgSO_4$, 0.10 ml of 12 mM NADP and 0.10 ml of 16 mM ATP.

(d) Measurement of Pyruvate and Lactate: The reaction of LDH is used as the indicator system, in which the change in the absorbancy of NADH at 334, 340 or 366 nm can be measured

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*Glucose-UV Method-Kit, Boehringer-Mannheim Test Combination Reagent.

photometrically.*

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

The aqueous humor is diluted x100 for lactate assay and x20 for pyruvate to obtain an accurate reading of optical density.

The quantity of metabolites is expressed as mg% as well as micromoles per ml of aqueous humor.

3. Results

(a) Relationship of TRE to Storage Time: Fig. 1 shows the curve of TRE versus storage time in eyes with aqueous is asymptotic with the horizontal axis, the temperature reversal reaching approximately 13% at 138 hours storage. A marked decline in the TRE was present between 0 and 24 hours of storage time, showing a levelling off at about 24 hours.

Fig. 1 also shows the curve of the TRE versus storage time in eyes without aqueous. This curve is hyperbolic to that of the TRE versus storage time in eyes with aqueous, and there is an asymptote at + 13% of TRE up to 138 hours of storage time. As the graph shows, a reverse TRE was present between 0 and 24 hours storage time, where-

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*LDH-UV Method-Kit, Boehringer-Mannheim Test Combination Reagent.

as a positive TRE was present thereafter.

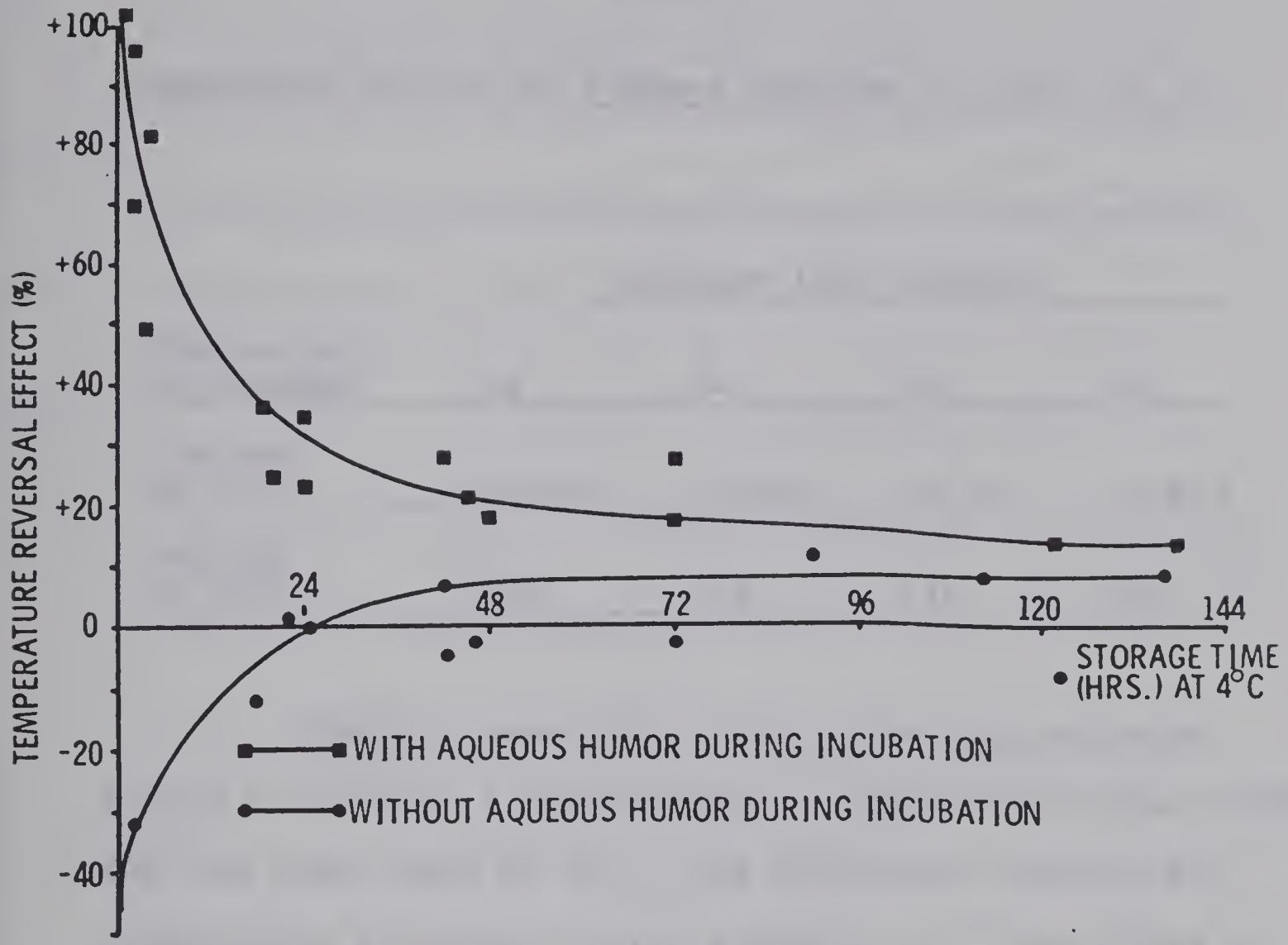


Fig. 1. Temperature Reversal Effect Versus Storage Time at 4°C.

In other words, the eyes without aqueous stored up to 24 hours at 4°C increase further in corneal thickness rather than showing a decrease during incubation at 37°C, while those stored longer than 24 hours show a reduction in corneal thickness.

TABLE I

Comparison of TRE (%) between Storage at 23°C and 4°C

	Storage time (hours)			
Temperature of Storage	16	22	40	46
Storage at 23°C	44.8%	24.8%	8.7%	1.83%
Storage at 4°C	40%	34%	23%	22%

Table I shows the TRE for the four cat eyes stored at 23°C in a moist chamber compared with eyes stored for the same times at 4°C. The difference between the temperature reversal in eyes stored at 23°C and those at 4°C is more marked the longer the storage.

(b) The Relationship of Metabolites to Storage Time: The glucose level in the aqueous humor of a cat falls rapidly to approximately 1/3 (44.2 mg%/158 mg% = 28%) of the fresh aqueous level and then gradually to almost 0 after 5 days storage (Fig. 2) at 4°C.

The levels of lactate and pyruvate after storage (but before incubation) are shown in Fig. 3. Although there was a marked scattering of values between 24 and 96 hours, the overall change was apparent. Two different

FIG. 2.: RELATIONSHIP OF GLUCOSE LEVEL IN AQUEOUS HUMOR TO STORAGE TIME AT 4° C

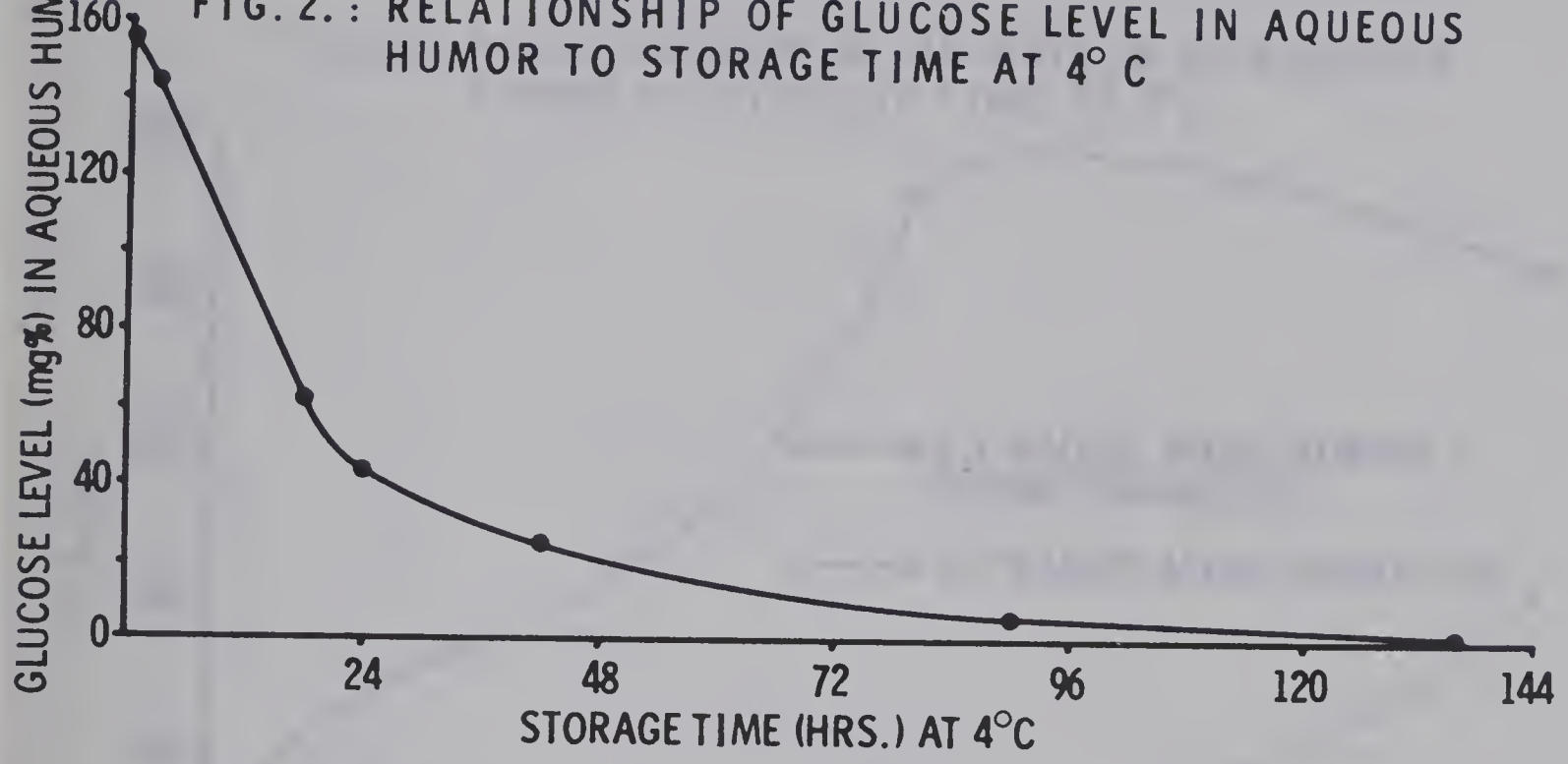
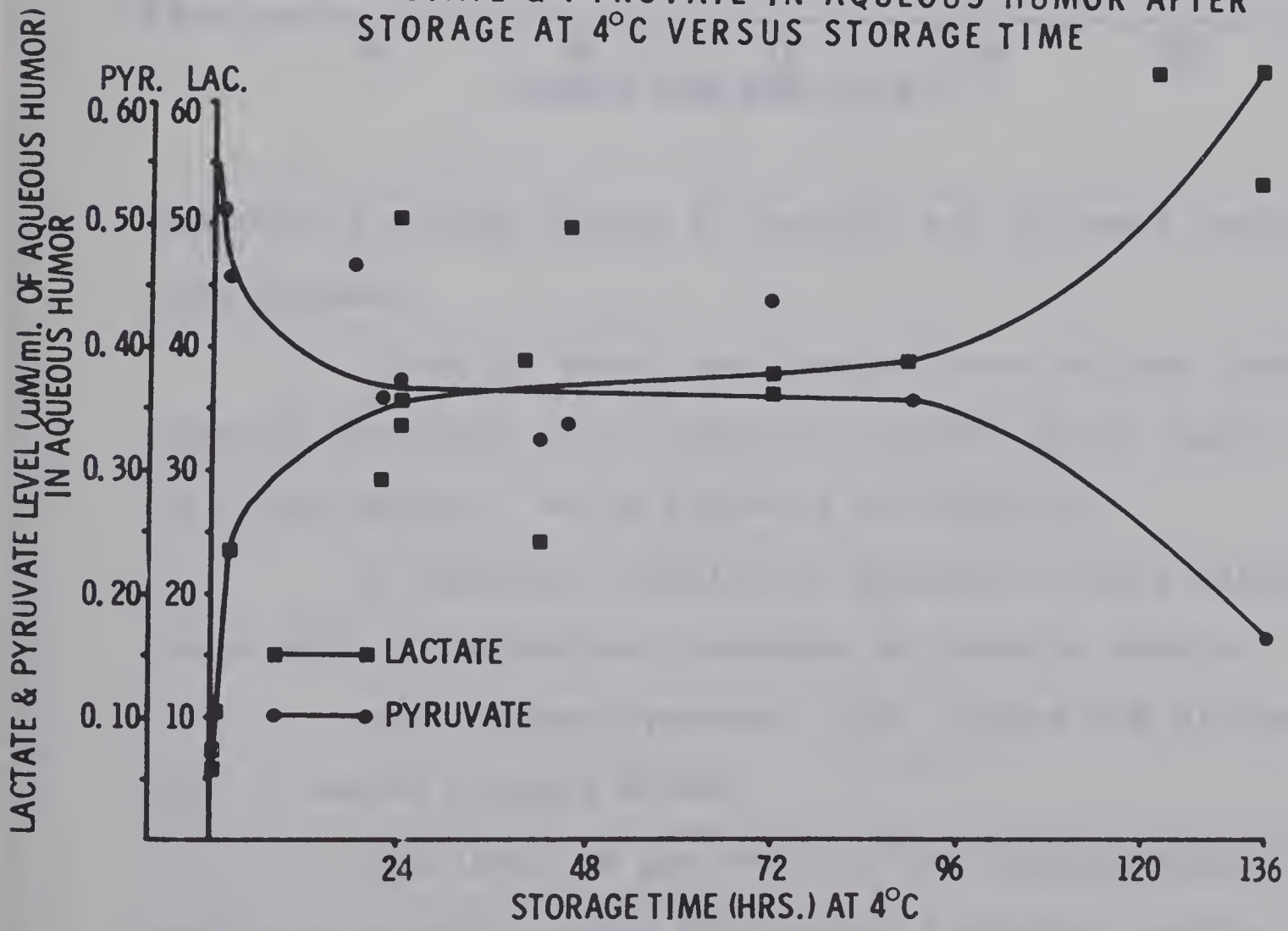


FIG. 3.: LACTATE & PYRUVATE IN AQUEOUS HUMOR AFTER STORAGE AT 4°C VERSUS STORAGE TIME



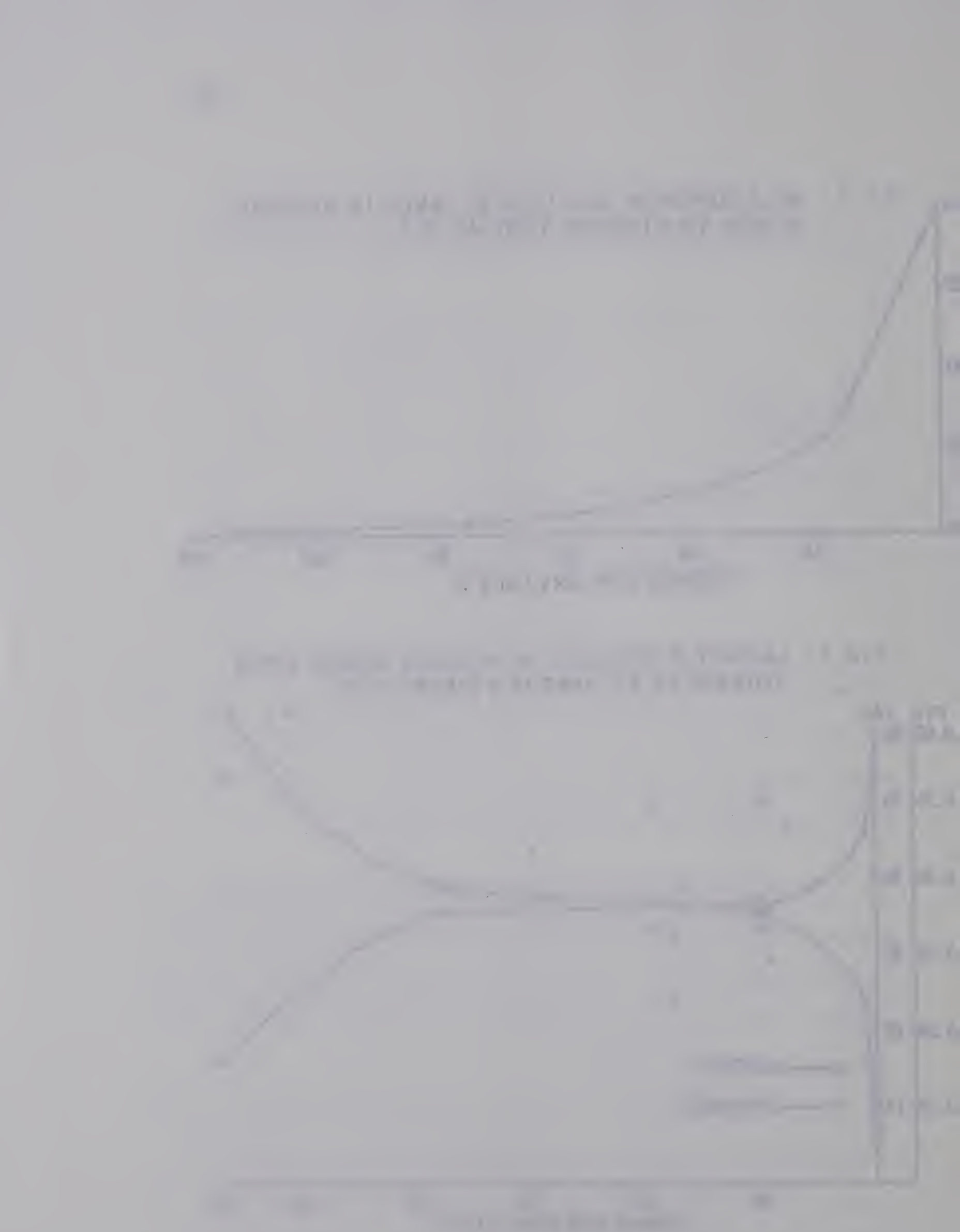
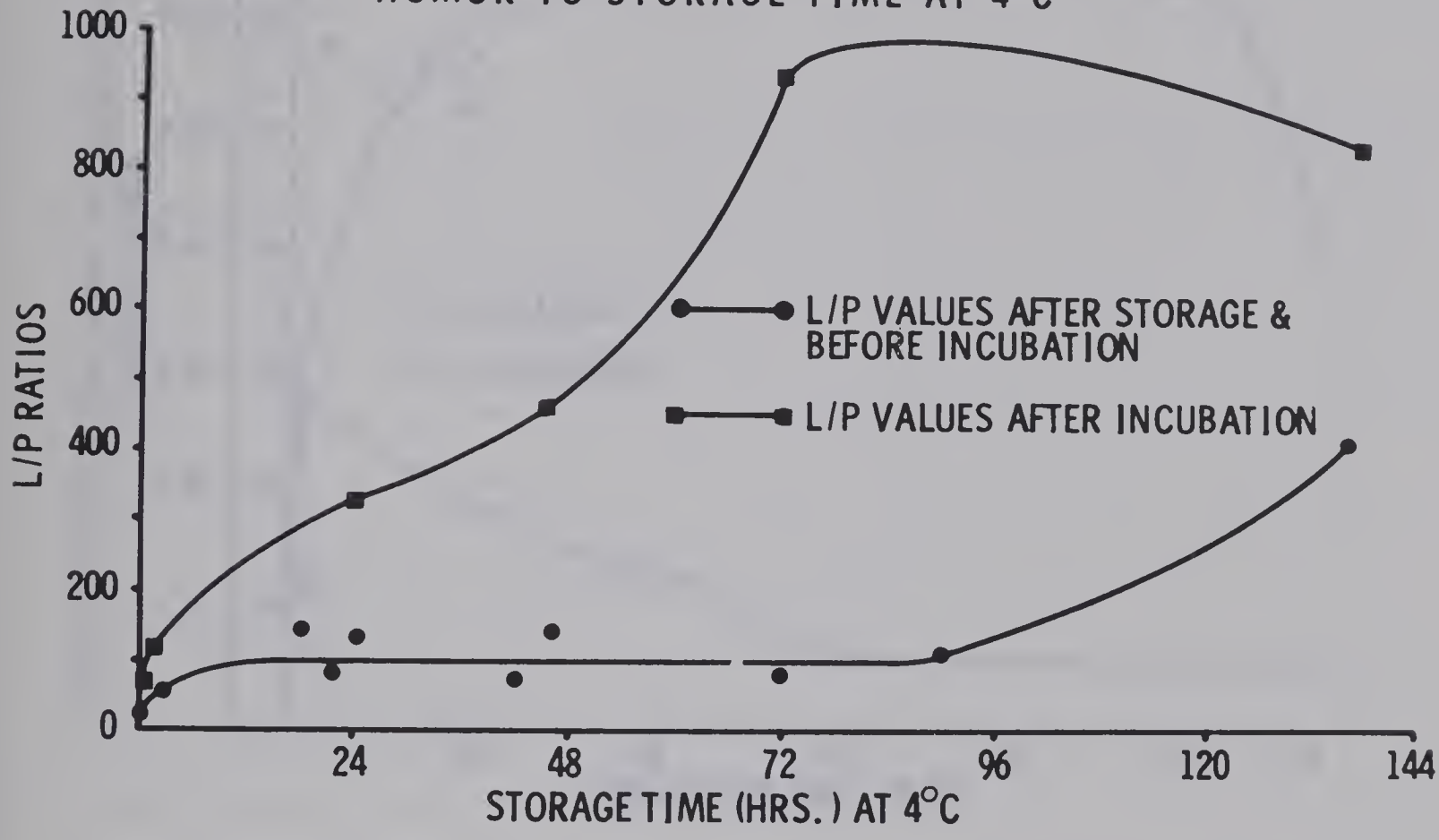


FIG.4.: RELATIONSHIP OF L/P RATIOS IN AQUEOUS HUMOR TO STORAGE TIME AT 4°C



periods of marked change in lactate and pyruvate levels were present.

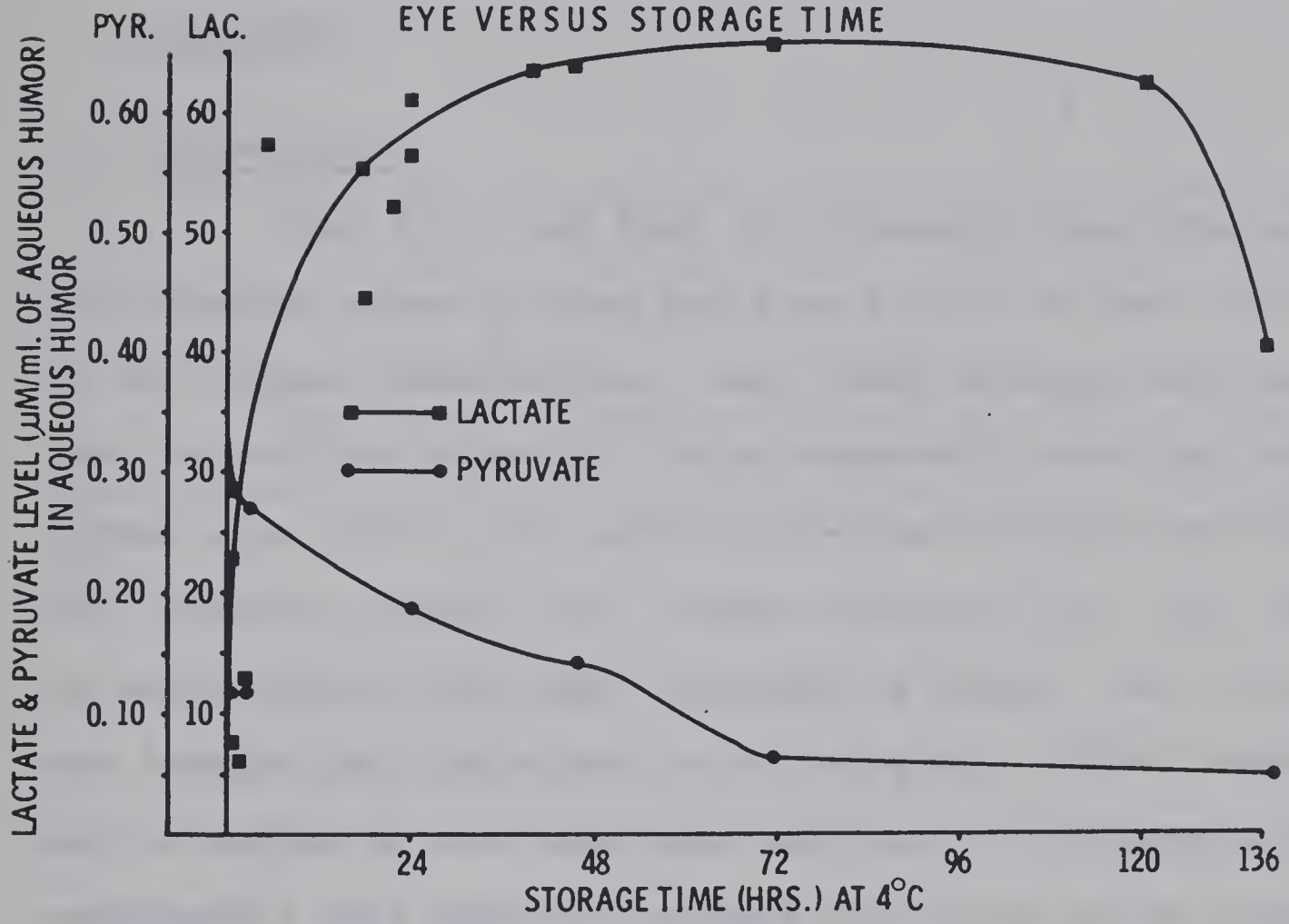
Firstly, after enucleation, the lactate level in aqueous increases in 24 hours to a level three times that in fresh aqueous, while pyruvate is dropping.

A secondary decline of pyruvate occurs after 96 hours with a concomitant increase in lactate levels.

The lactate/pyruvate (L/P) ratios are plotted in Fig. 4 versus storage stime.

The level of pyruvate in the aqueous after incubation of a refrigerated eye showed a gradual decline with

FIG. 5.: LACTATE & PYRUVATE IN AQUEOUS HUMOR AFTER INCUBATION AT 37°C OF REFRIGERATED EYE VERSUS STORAGE TIME



increased storage time, whereas that of lactate showed a rapid increase in 24 hours (Fig. 5), producing very high L/P ratios after 24 hours, up to 5 days. The L/P ratio at 5 days storage is more than 50 times that in fresh aqueous, while during that time of storage at 4°C it remains constant at around 100, i.e., 5 times that in fresh aqueous.

The L/P ratio for the eye after incubation declines after 5 days storage, accompanied by a fall of lactate, whereas the L/P ratio after storage and before incubation begins to increase.

B. LDH AND ITS ISOZYME AS A MEASURE OF CORNEAL ENDOTHELIAL VIABILITY

1. Introduction

Jans (47), and Kirk (57) recently used the uptake of Lissamine green by dead cells as a test of the viability of cat corneal endothelium. Many other methods have been used to evaluate viability using supravital staining with trypan blue (109, 110), para-nitro-blue tetrazolium (NBT) for oxidative enzymes (6), tissue culture (109, 111), and an embryonation test (16). In most of these, the cornea was damaged for future use in a transplant. This research was attempted in the hope that analysis of the aqueous for endothelial cell specific enzymes will prove to be a practical tool for determining corneal viability without damaging the cornea for future use.

2. Methods and Materials

(a) Collection of Eyes and Aqueous Humor: Human aqueous was collected from cataract cases during extraction and from intact eye-bank eyes which were not used for corneal transplants. Human aqueous was also obtained from post-mortems.

Cat eyes used for aqueous analysis were enucleated from decerebrated or choline-anesthetized animals while still living or immediately after death.

The cat eyes used for a quantitative measurement of LDH activity in the aqueous following mechanical damage to the corneal endothelium were from cats aged two years or over, and weighing 2.8 - 4.3 Kg sacrificed by intracardiac injection of nembutal. The eyes were stored under standard eye-bank conditions for varying periods.

Only aqueous which was macroscopically clear of haemorrhage and which was colorless was used for the enzyme assay.

(b) Preparation of Tissue Extracts:

(i) Endothelial Fraction: After scraping off all of the epithelium, the cornea was totally excised except for approximately 1 mm at the limbus. The excised cornea was then weighed on a Mettler balance, and frozen by Kryokwik* sprayed on the endothelial surface. The endothelium was scraped off the posterior surface of the frozen cornea with a scalpel, and the tissue-powder placed immediately in 1.0 ml of redistilled water. This suspension was homogenized in a glass-to-glass homogenizer and the resulting homogenate centrifuged in a general laboratory centrifuge (Sorvall) at 3000 r.p.m. for 10 min. The wet weight of the endothelial fraction was estimated by the difference between the

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*A mixture of fluorinated hydrocarbons; International Equipment Company.

weight of the stromal fraction before and after the removal of the endothelium.

(ii) Stromal Fraction: After weighing, the stroma was placed in 2.0 ml of redistilled water and homogenized in a glass-to-glass homogenizer for approximately 15 min until complete maceration of the tissue occurred. The centrifugation procedure was identical to that for the endothelial fraction.

(iii) Epithelial Fraction: The scraped tissue-powder of epithelium was placed in 1.0 ml of redistilled water. The procedure for homogenization and centrifugation was identical to that for the endothelial fraction.

(iv) Iris and Ciliary Body: The iris and ciliary body were grasped from within after transsecting the globe and removing the lens, and stripped away from the ocular wall. This tissue was then homogenized in 2.0 ml of redistilled water.

(v) Lens Extract: A tissue extract of lens was also obtained using 2.0 ml of redistilled water and the same technique as for the homogenate of the other tissue.

(c) Measurement of Enzymes:

(i) LDH and Its Isozymes: The total LDH activity was determined using a Beckman DB-G spectrophotometer. In this procedure, pyruvate and NADH were converted into lactate and

NAD^+ with a concomitant decrease of absorbancy of NADH. Boehringer-Mannheim Test Combination Reagents were used in a total volume of 3 ml, 0.1 ml consisted of aqueous humor and the remainder of 0.3 mM pyruvate. For eyes stored longer, the aqueous had to be diluted x10 or x100 to obtain an accurate reading of optical density since LDH activity became fairly high. The rate of decrease in absorbancy was measured at 340 nm or 366 nm at 25°C and pH 7.5, and expressed as International Units. One unit corresponds to the amount of enzyme which converts 1 micromole of substrate per minute.

The isozyme level was calculated by multiplying its ratio in densitogram by the amount of total LDH.

(ii) G-6-PDH: G-6-PDH converts G-6-P and $NADP^+$ into 6-PG and NADPH with a concomitant increase in optical density of NADPH.

$$\text{G-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+$$

This analysis was carried out as follows: 0.5 ml of aqueous humor was diluted with 0.5 ml of redistilled water. To that was added 2 ml of 0.05 M triethanolamine buffer, then, 0.10 ml of 0.01 m $NADP^+$ and 0.05 ml of 0.03 M G-6-P, in this order.

The rate of increase in absorbancy was measured at 340 nm or 366 nm at 25°C. The mean absorbancy differ-

ence was calculated from the constantly increasing period of optical density which developed after a bit of a lag period. The lag period of G-6-PDH enzyme reaction in this aqueous humor assay system was more marked with low enzyme than with high enzyme activity.

(iii) ICDH: NADP-dependent ICDH converts isocitrate and $NADP^+$ into oxalosuccinate and NADPH in the presence of Mg^{++}.

$$\text{Isocitrate} + NADP^+ \xrightleftharpoons{\text{ICDH}} \text{Oxalosuccinate} + NADPH + H^+$$

The above reaction of ICDH was used for the measurement of its activity. 0.50 ml of aqueous humor was mixed in 2.50 ml of 4.6 mM DL-isocitrate in 0.1 M triethanolamine buffer, pH 7.5. To this mixture, 0.10 ml of the solution of 9.1 mM $NADP^+$ and 0.12 M $MnSO_4$ was added. The rate of increase in absorbancy of NADPH revealed a linearity from the beginning of the reaction.

(d) Mechanical Damage of Endothelium: A scleral fit contact lens for the cat eye was molded, and engraved with a grid consisting of 5 vertical and 5 horizontal lines. The contact lens was placed on the eye and a 25 G needle was inserted through the cornea at the limbus. Using the grid as a guide, scratches were made on the endothelium corresponding to lines 1, 3, 5, 7 and 9 to produce a constant area of damage.

The level of LDH and its isozymes in the aqueous was measured in the damaged eye and in a control eye without endothelial damage.

3. Results

(a) Base-Line Values in LDH and Its Isozymes: Table II shows the base line values of LDH in the fresh aqueous of normal human and cat eyes.

TABLE II

Total Activity of LDH in Fresh Aqueous Humor

	No of Assays	Mean (mU)	Standard Error of Means
Human	4	26.5	1.97
Cat	4	19.6	0.95

The LDH-isozyme distribution in fresh human aqueous humor is quite different from that of the cat as shown in Table III and Fig. 6.

The H-type was more predominant in the cat and the M-type more predominant in the human.

TABLE III

% Isozymes of LDH in Fresh Aqueous Humor

Isozyme Fractions	Human		Cat	
	Case 1	Case 2	Case 1	Case 2
1 (H-4)	10(%)	18(%)	27(%)	33(%)
2 (H-3 M-1)	10	27	42	33
3 (H-2 M-2)	24	21	23	20
4 (H-1 M-3)	50	9	8	13
5 (M-4)	5	26	-	-

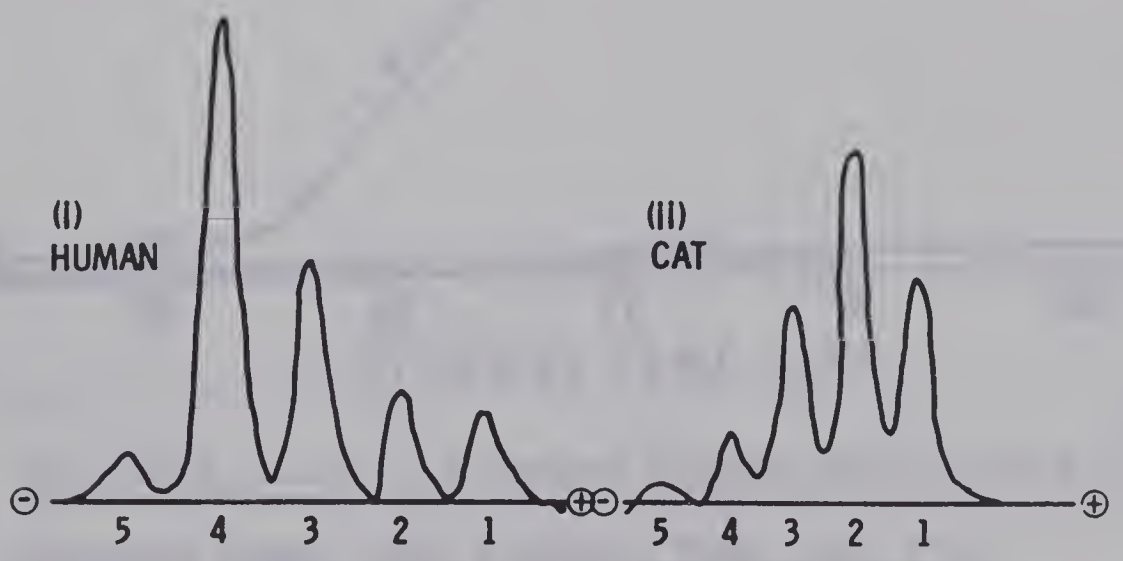


Fig. 6. Sample Densitogram of the LDH-Isozymes in Aqueous Humor

(b) Relationship of Enzyme Activities in Cat and Human Aqueous to Storage Time: Fig. 7 shows the change in levels of LDH, G-6-PDH and ICDH in cat aqueous plotted against storage time. At 4°C storage, ICDH and G-6-PDH were present in the aqueous in measurable amounts after 22 hours in

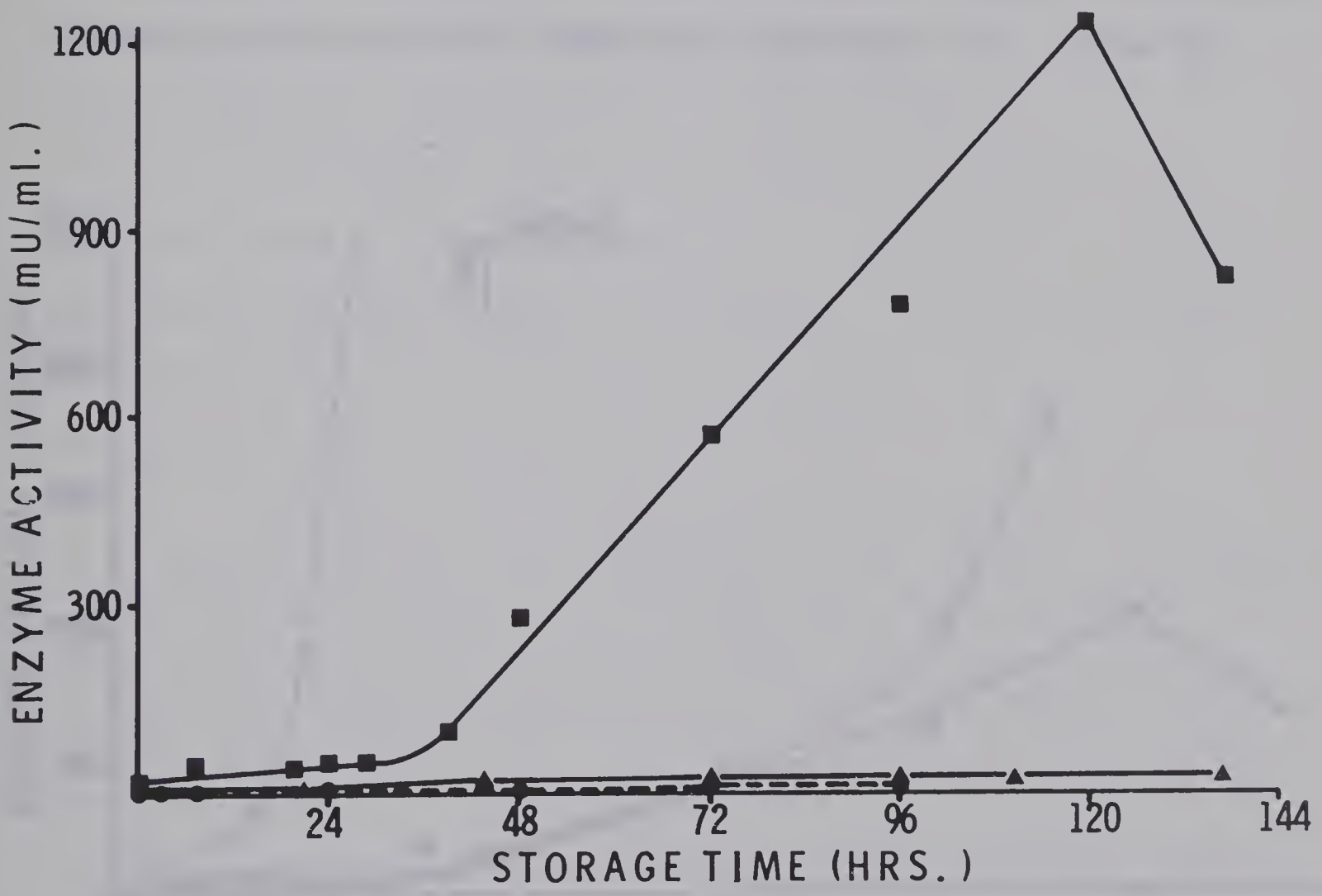
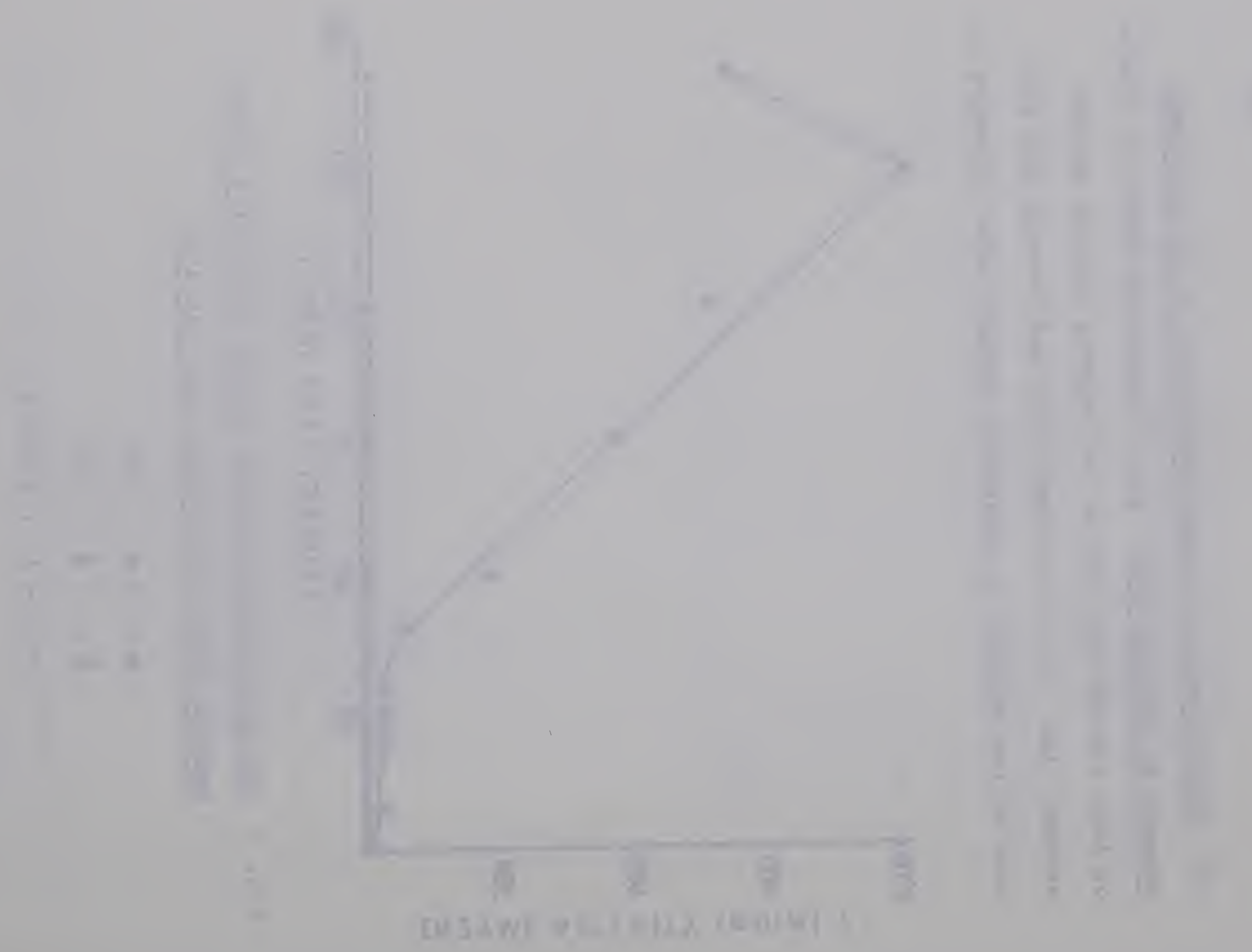


Fig. 7. The Relationship between Enzyme Activities in Cat Aqueous Humor and Storage Time (at 4°C)

■ —— ■ ; LDH

▲ —— ▲ ; ICDH

● —— ● ; G-6-PDH



the former, and after 72 hours in the latter. The activity of both G-6-PDH and ICDH in the aqueous humor was then constant at a very low level for the entire prolonged storage period.

LDH, on the other hand, showed a slight increase in activity in the aqueous up to 24 hours storage in a moist chamber at 4°C in both human and cat (Fig. 8). After 24

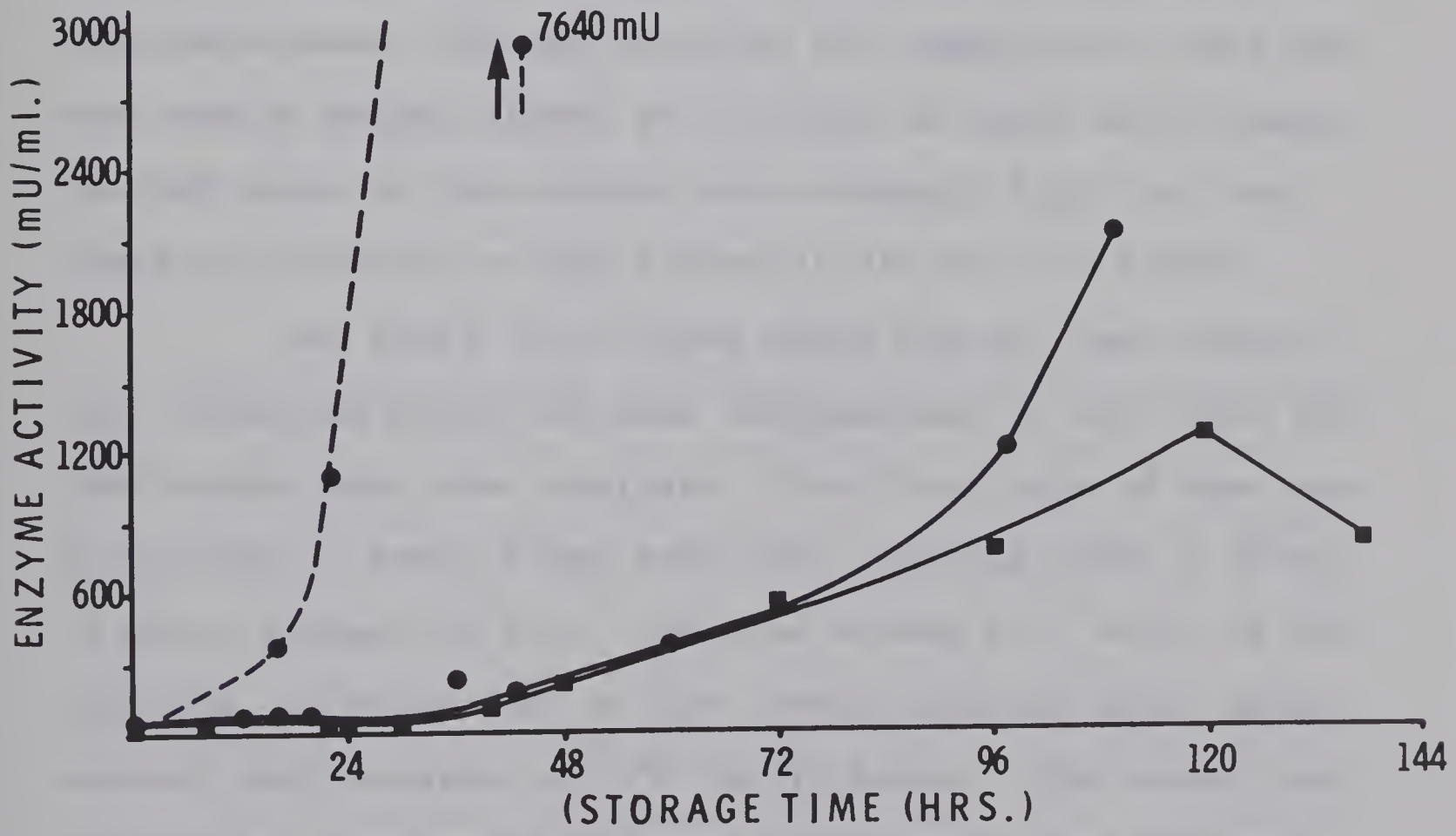


Fig. 8. The Relationship between LDH levels and Storage Time

●——● ; Human Aqueous in Eyes Stored at 4°C

■——■ ; Cat Aqueous in Eyes Stored at 4°C

●- - - -● ; Cat Aqueous in Eyes Stored at 23°C

hours, the increase in activity in both species was marked. Human aqueous, however, showed a more rapid increase in LDH activity than cat aqueous after 60 hours of refrigeration (Fig. 8), in the human and cat eye, the cat being more resistant to damage. As shown in Fig. 8, with storage at room temperature (+23°C) the LDH level in cat aqueous humor increased more rapidly than with storage at 4°C up to 16 hours, and very rapidly after 16 hours storage.

One frozen cat eye was obtained incidental to this experiment, and was analyzed for comparison. This eye was from an animal frozen at -7°C for 12 hours after death. The LDH level in the aqueous was extremely high i.e. ten times the level of an eye stored at 4°C for 122 hours.

Two pairs of cat eyes which had not been enucleated fresh and which had been refrigerated in situ with the dead animal were also analyzed. The first pair of eyes was enucleated 12 hours after death and analyzed after 2 hours of moist storage at 4°C. The eyes showed 41.1 mU/ml of LDH activity, corresponding to the levels obtained after moist chamber refrigeration at 4°C for 15 hours. The second pair of eyes was enucleated 28 hours after death, and analyzed immediately. The LDH activity was 182 mU/ml in one eye and 115 mU/ml in the other. This level corresponds to the LDH level obtained in an eye stored for 35 hours in a moist chamber at 4°C.

(c) LDH and Its Isozymes after Endothelial Damage: After mechanical damage to the corneal endothelium, the LDH level in the aqueous humor increases in direct proportion to the amount of endothelial damage. An increase in LDH-1-isozyme level was also noted (Fig. 9). This was shown by the difference in levels from the undamaged control eye.

The same series of cat eyes were also assayed for LDH-isozymes by densitometry following electrophoretic separation. Endothelial damage in the cat produced an increase in LDH-1-isozyme, but in the human an increase in LDH-5-isozyme (Fig. 10). This suggests a species variation in the LDH isozyme pattern of the corneal endothelium in damaged the human and cat.

(d) Relationship of LDH-1-Isozyme in Cat Aqueous to Storage Time: Early in storage, the predominant increase was in the LDH-1-isozyme; increased levels of LDH-5-isozyme appear later. This suggests that the increase in total LDH in the aqueous originates mainly from the corneal endothelium in early storage, reflecting that LDH-1 was the isozyme mainly increased in endothelial damage (Fig. 10). The appearance of LDH-5 after later storage reflects damage to the other surrounding tissues.

The relationship of LDH-1-isozyme to storage time is shown as a graph in Fig. 11. This graph shows a more rapid rise at 24 hours than the rise of total LDH at this

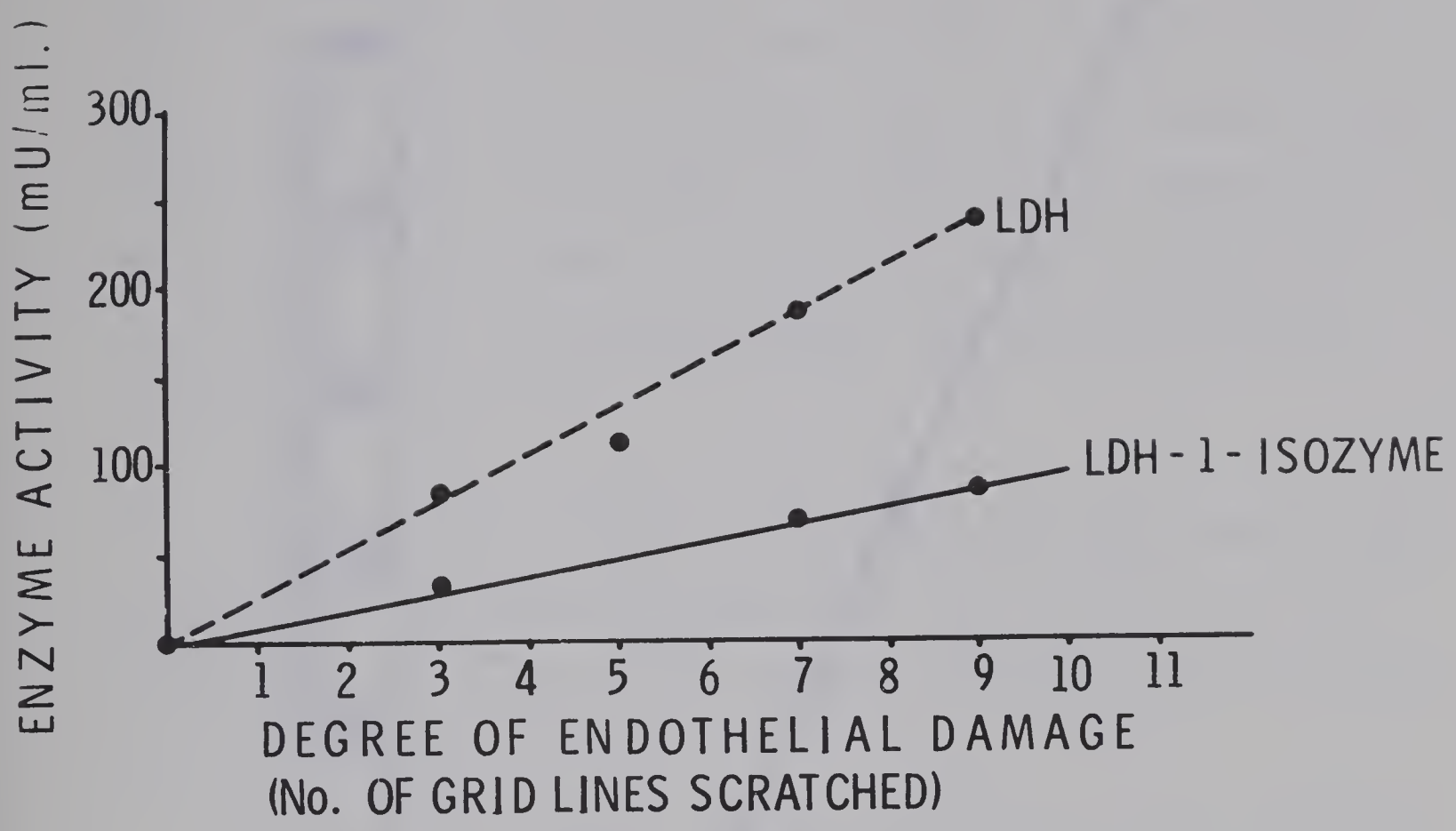


Fig. 9. The Relationship of LDH and Its 1-Isozyme in Cat Aqueous Humor to Endothelial Damage

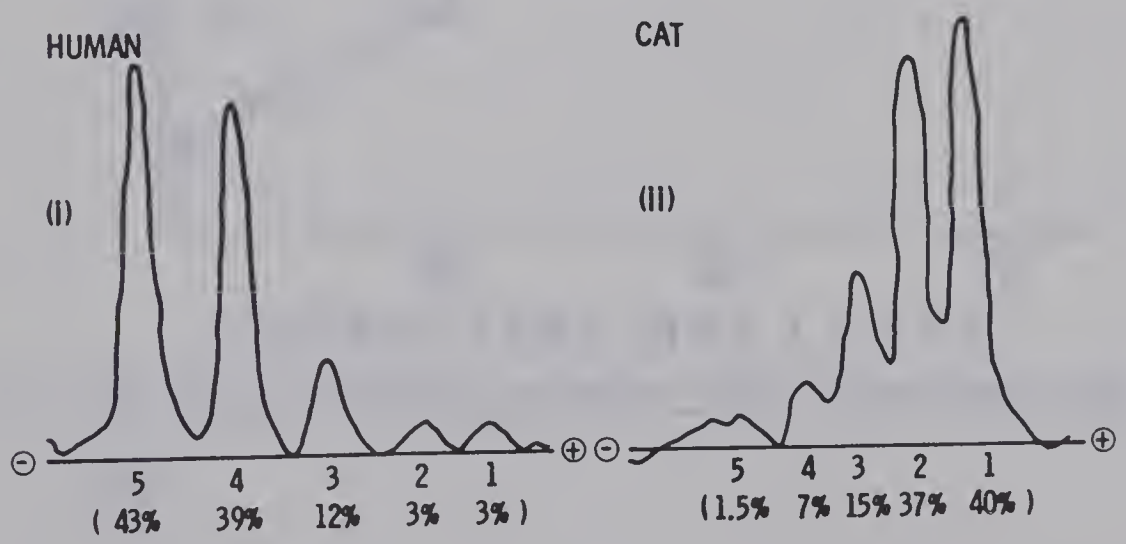
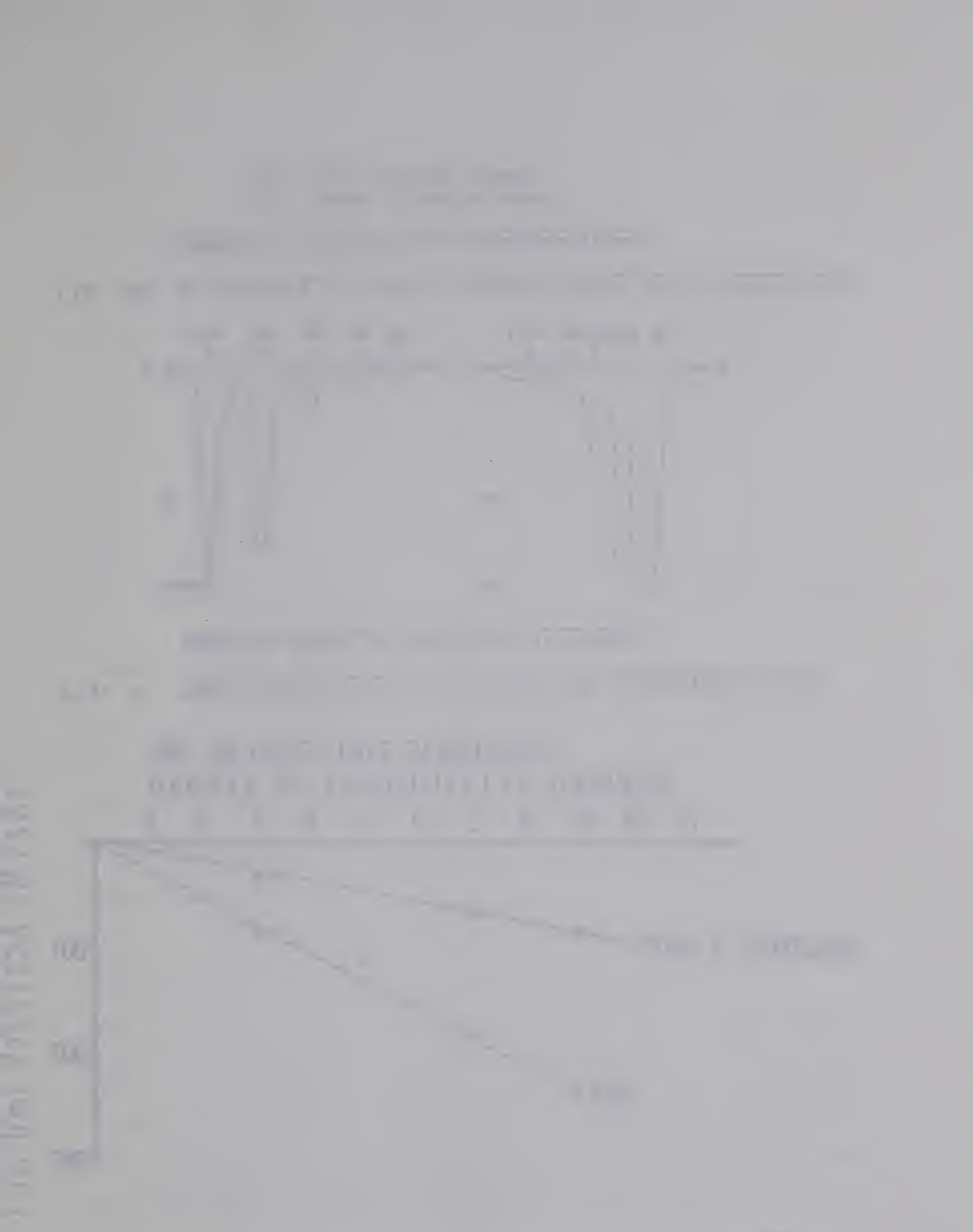


Fig. 10. % Isozymes of LDH in Aqueous Humor after Mechanical Damage to Endothelium (Densitograms)

(i) Human Aqueous Humor
(ii) Cat Aqueous Humor



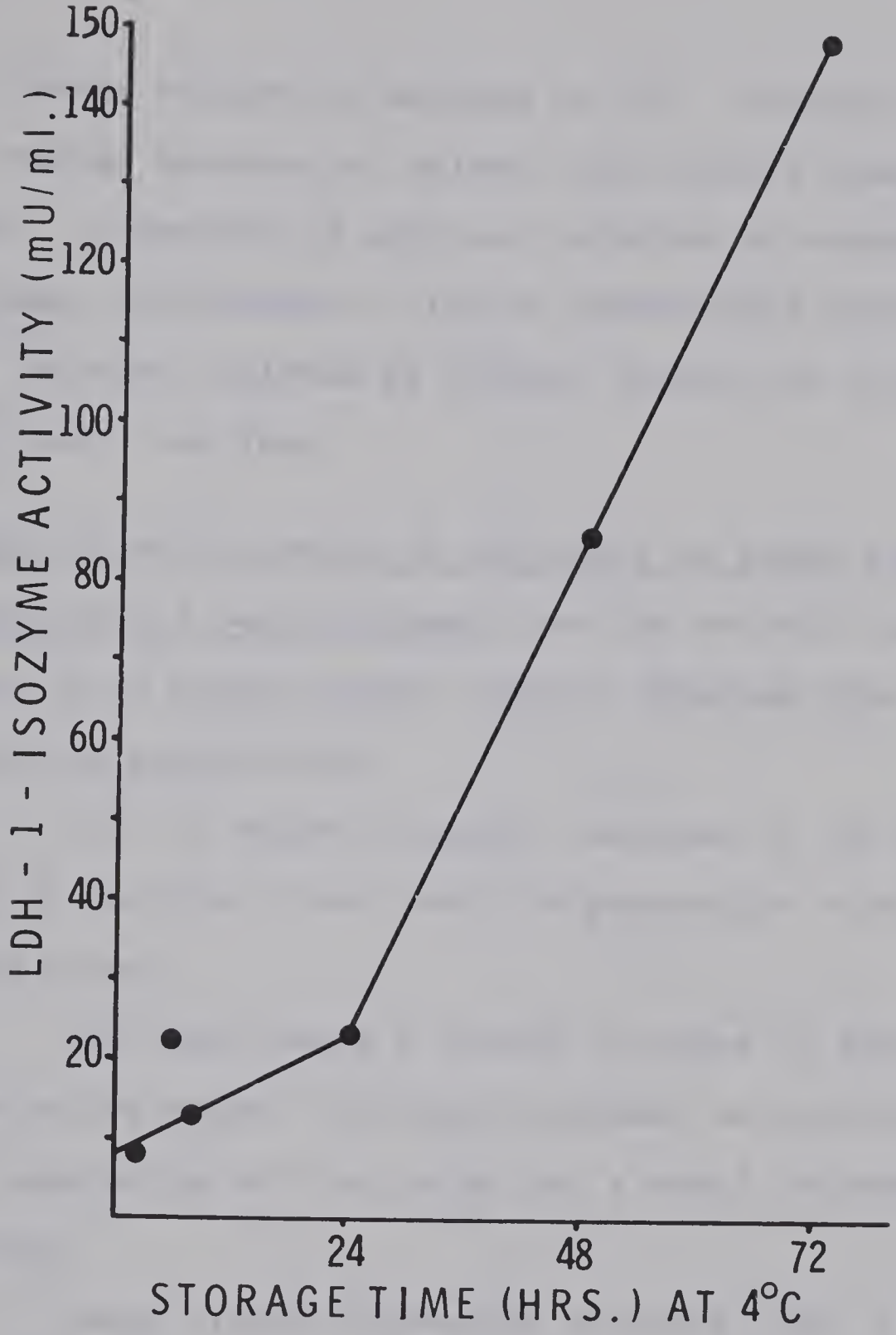


Fig. 11. The Relationship between LDH-1-Isozyme and Storage Time

point (Fig. 7).

(e) The Relationship of LDH in Human Aqueous and Adjacent Tissues to Storage Time: Fig. 12 shows the different pattern of changes of LDH levels in human aqueous and in adja-

cent tissues related to storage at 4°C. Although there were a marked scatters of values, the overall changes were apparent. A decline of LDH was, occurred in endothelium and stroma, an increase of LDH in aqueous and epithelium and an increase followed by gradual decline in iris and citiary body, and lens.

(f) The Borderline Value of LDH-Level in Human Aqueous for the Selection of Donor Corneas: The LDH activity has been analyzed in 70 human aqueous samples obtained from cataract patients and postmortems.

Fig. 13 shows a regular increase in LDH activity related to the time since death in postmortem cases (measured in hours).

The curve shows a change in slope at approximately 20 hours after death: A slight increase in activity occurs in the aqueous up to this point and a rapid increase after this point.

Using linear regression analysis (Fig. 14) the same series of samples before 6 hours since death was studied for the influence of age on the LDH level in aqueous.

If the LDH level were a function of age, subjects of different ages would have different means of LDH-level and the mean for the material as a whole would be meaningless.

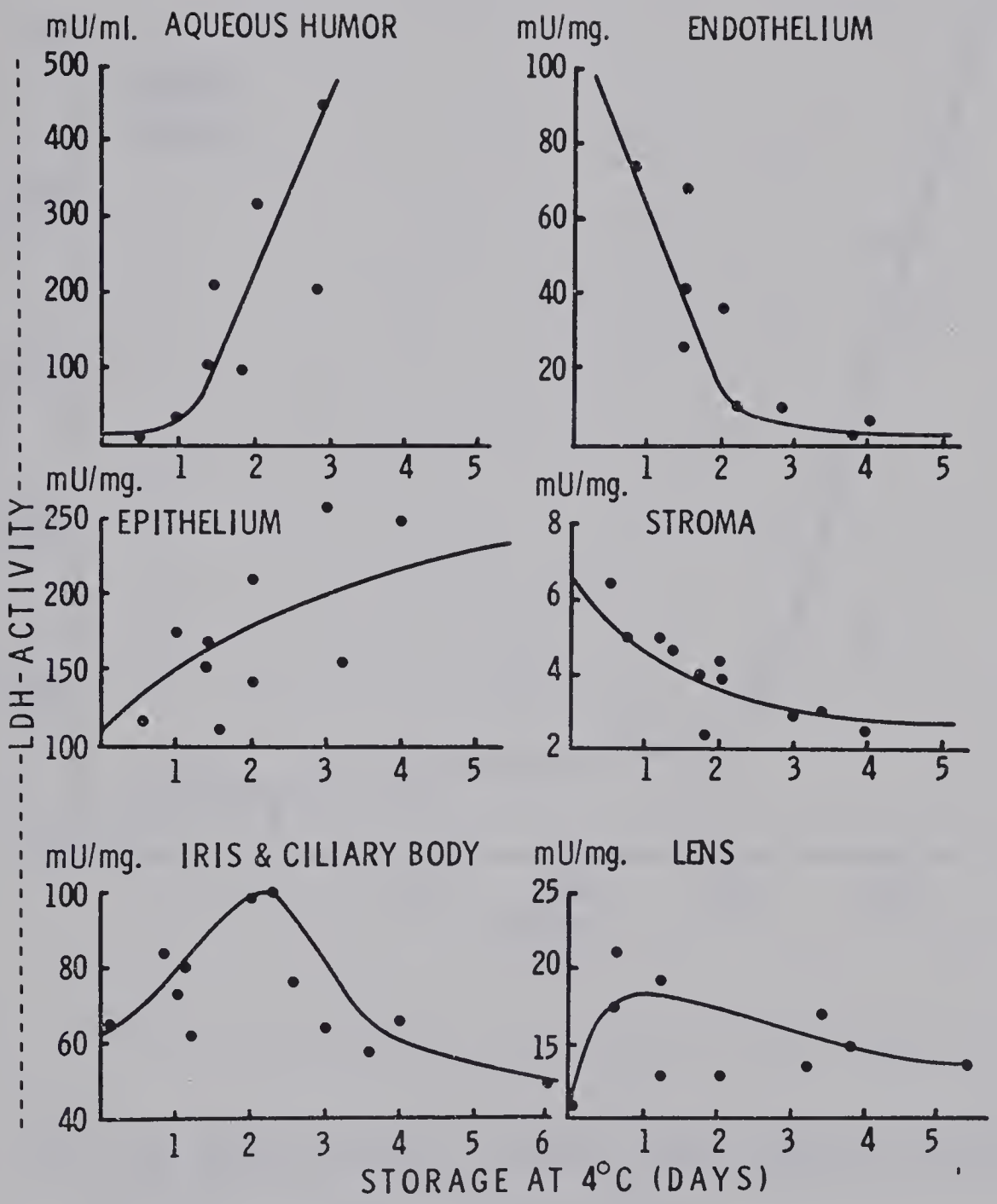


Fig. 12. The Relationship of LDH in Human Aqueous and Adjacent Tissues to Storage Time

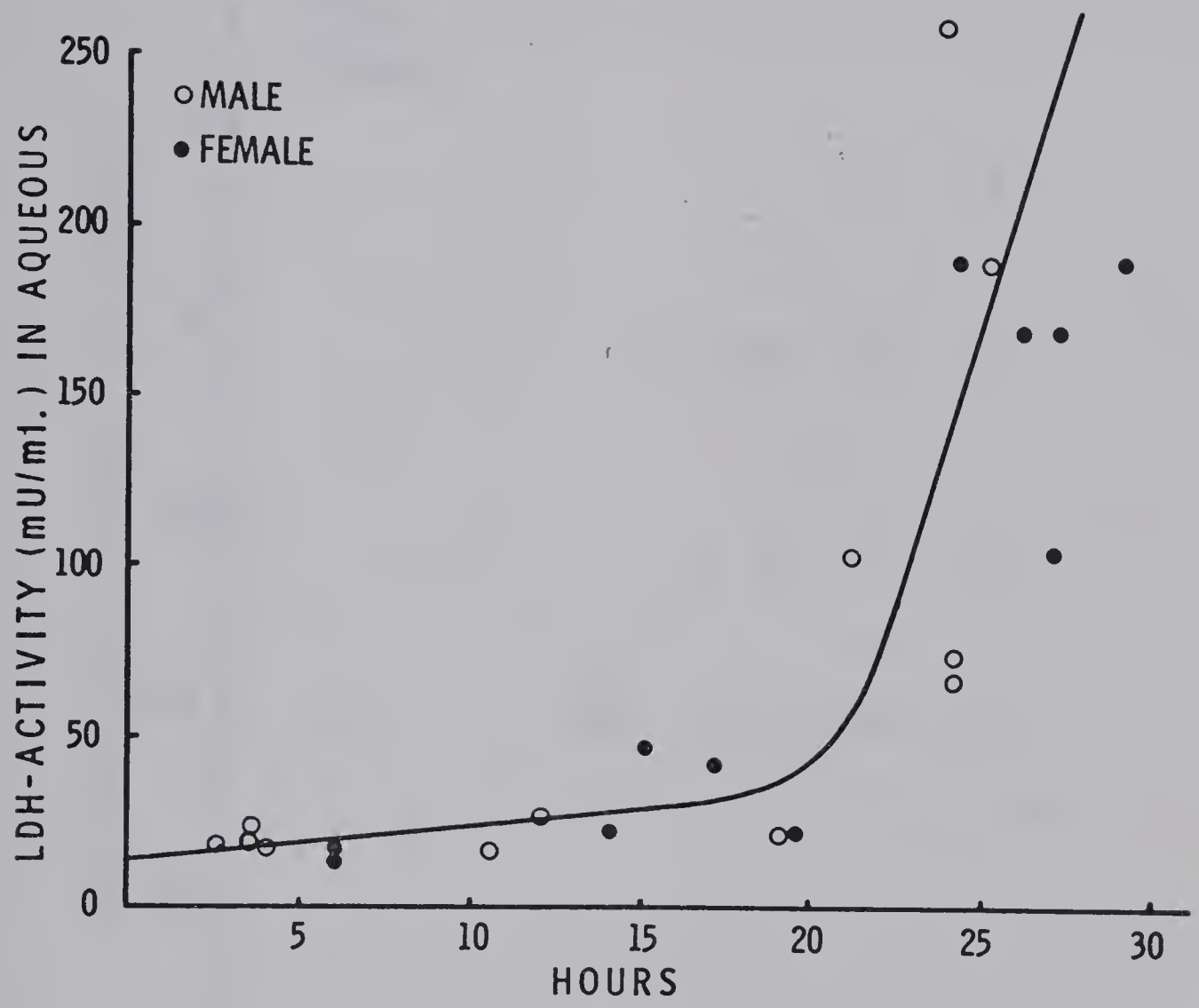


Fig. 13. The Relationship between LDH Levels and Time since Death in Human Postmortems

The regression as a function of age of the LDH-level in aqueous(y) was $y = 20.77 + 0.13\,x$ for the whole group. Sex was not found to have any effect. The correlation of LDH to age was not significant ($r = 0.31$, $p < 0.10$).

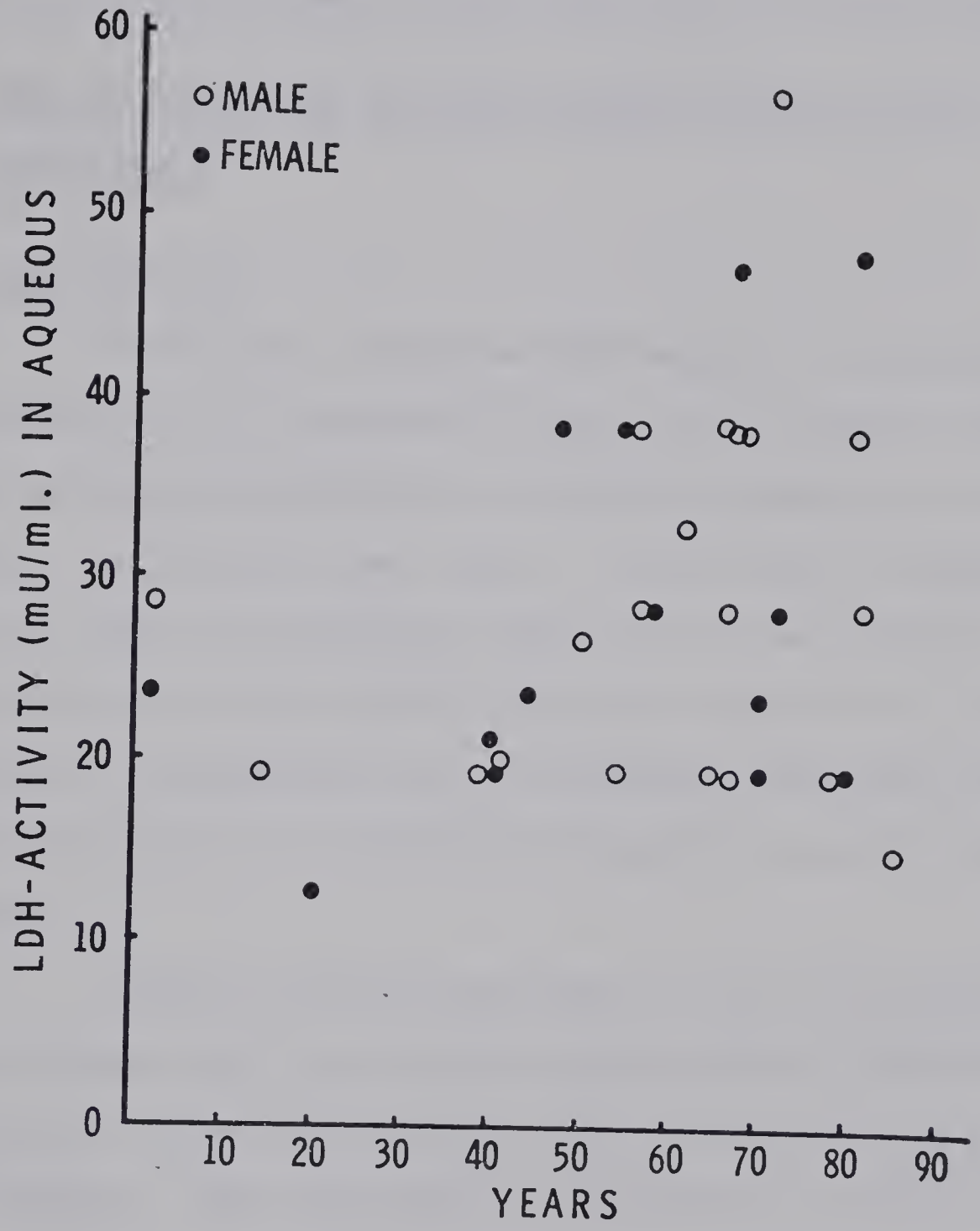


Fig. 14. The Relationship of LDH Levels in Aqueous to the Age of Donor

The LDH level averaged for the whole group was 28.06 ± 10.11 (S.D.). A percent probability of 95% is

28.06 ± 20.22 mU/ml (2 S.D.). If a probability of 99% is taken, the upper limit of LDH-level will be 53.34 mU/ml.

C. SPECIES VARIATION IN LDH ISOZYME PATTERN OF THE CORNEAL ENDOTHELIUM

1. Introduction

During the study on LDH isozymes of aqueous, it was shown that an increase in LDH_1 in cat aqueous was produced by corneal endothelial damage, in contrast to an increase in LDH_5 in human eyes. It has also re-emphasized that the TRE is a metabolic function of the endothelium, with glucose in the aqueous playing a major role (Result III. A.). A secondary role is probably played by a substrate-reservoir in the cornea related to aerobic metabolic pathway.

Direct studies were made of the LDH isozyme pattern of human and cat corneal endothelium to determine the pattern of corneal endothelial LDH isozymes in the different species. This was done in an attempt to explain species difference in the vulnerability of corneal endothelial cells to damage when stored at 4°C.

2. Methods and Materials

(a) Preparation of Tissue Extracts: The procedures used to prepare tissue extracts of endothelial fractions and stromal fractions have been described in the previous sec-

tion (Section III.B.2.b.).

(b) Determination of LDH and Its Isozymes: The method used was identical to that for the aqueous humor.

3. Results

The LDH-isozyme patterns of human corneal endothelium differs from cat corneal endothelium as shown in Table IV and Fig. 15: Isozymes 1-3 are predominant in the latter, while LDH isozymes 3-5 are predominant in the former.

TABLE IV

% Isozymes of LDH in the Tissue Extract of the Corneal Endothelium

	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	Total LDH Activity (mU/mg of Wet Weight)
Human	1.5	6	32	42	19	98.7 ± 13.9
Cat	16	36	33	14	0.7	101.3 ± 5.8

The comparative isozyme patterns for the stroma are shown in Table V and Fig. 16. In both human and cat, the stroma shows predominant activity at LDH 3-5, the same pattern shown in human corneal endothelium.

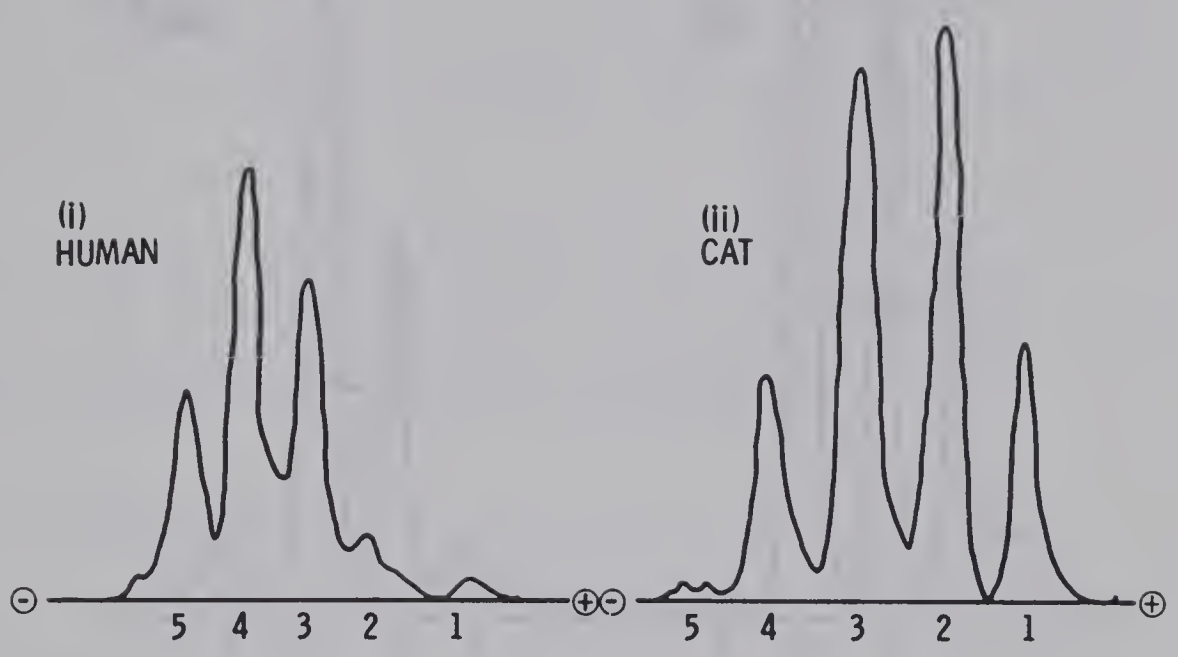


Fig. 15. Densitograms of the LDH-Isozymes in the Tissue Extract of the Corneal Endothelium (i) Human eye, (ii) Cat eye.

TABLE V

% Isozymes of LDH in the Tissue Extract of the Corneal Stroma

	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	Total LDH Activity (mU/mg of Wet Weight)
Human	2	6	29	58	5	5.80 ± 0.73
Cat	5	19	32	41	2	2.91 ± 1.26

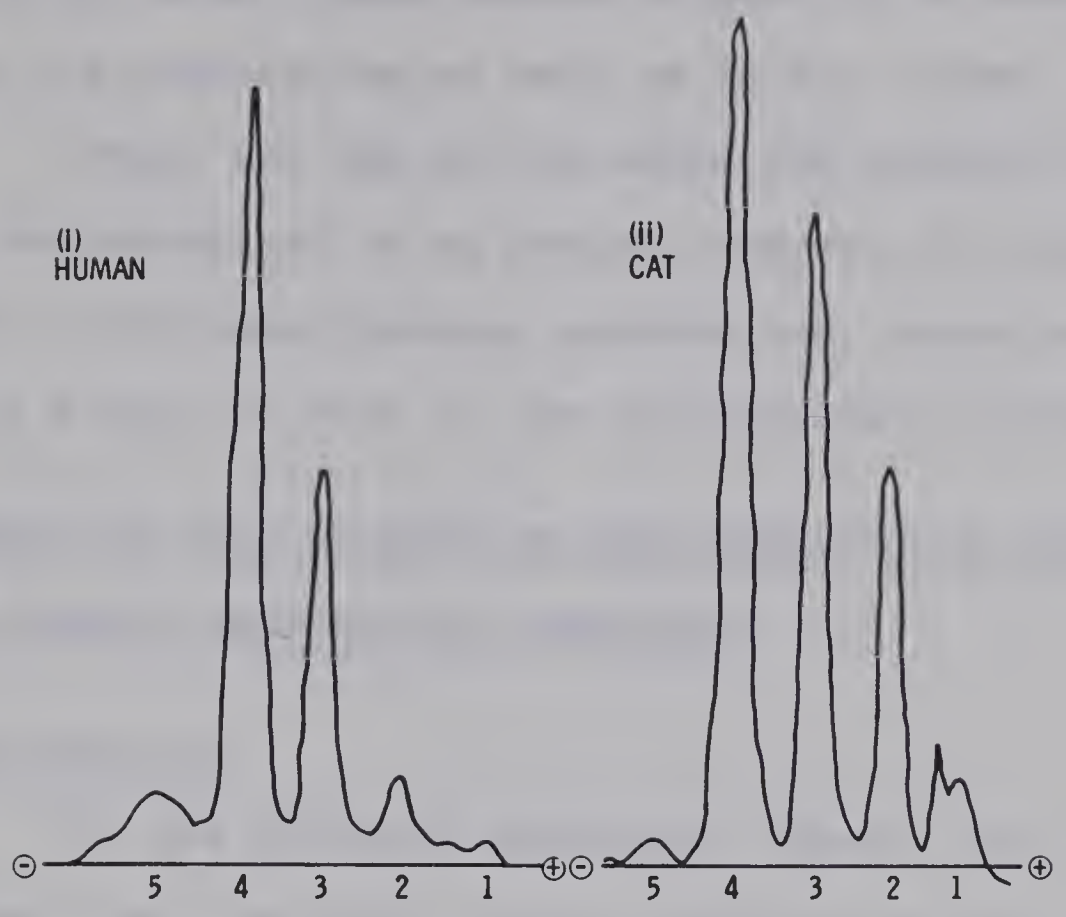


Fig. 16. Densitograms of the LDH-Isozymes in the Tissue Extract of the Corneal Stroma (i) Human eye, (ii) Cat eye.

The pattern for the cat stroma suggests more intense LDH-1 and LDH-2 than that of human stroma. This mild degree of difference can probably be attributed to possible contamination by the cat endothelium, which is characterized by a predominance of LDH 1-3. If one admits the possibility of such a contamination, the true picture for the cat stroma would certainly be the same M-type as that of human corneal stroma. It is clearly evident, therefore, that the cat endothelial isozyme pattern contrasts markedly with that of the corresponding stroma,

while in the adult human cornea M-type LDH is mainly present in the endothelium as well as in the stroma.

Thus, the LDH in the adult cat corneal endothelium is characterized by an H-type isozyme, in contrast to the LDH in the human corneal endothelium, characterized by the same M-type as that in the corresponding stroma.

D. EFFECT OF COLD STORAGE ON THE STABILITY OF LDH RELATED TO CORNEAL ENDOTHELIAL VIABILITY

1. Introduction

In the previous experiment (Result III. C.), it was shown that the human corneal endothelium was characterized by a M-type, in contrast to the cat corneal endothelium which shows a typical H-type. During studies on human corneal endothelium, a rapid inactivation of LDH activity was observed in short term storage of the tissue extracts at 4°C. Thus, it was decided to test the validity of the findings in the experiment C. by estimating a cold stability index of LDH based on the ratio of residual activity to initial activity in terms of the percentage residual of total LDH after long term storage at 4°C.

2. Methods and Materials

(a) Collection of Samples: The samples consisted of various tissue extracts from the anterior segment of the eye and the aqueous of human and cat. The method employed for

preparation of tissue extracts has been described in the previous section (Section III.B.2.b.).

Only the samples, on which isozymic distributions had been determined, were used for the calculation of percentage residual after long-term storage.

(b) Stability Index of LDH: The stability index of the enzyme following storage in vitro was calculated in terms of the percentage residual of the total activity:

$$\%age\ Residual = \frac{Residual\ Activity\ (A_R)}{Initial\ Activity\ (A_I)} \times 100$$

The initial activity represents the level of the total activity in freshly prepared tissue extracts and the residual activity corresponds to the remaining activity of the enzyme in the sample after storage.

The effect of the storage temperature on the stability of LDH was investigated at 4°C and 23°C for long-term storage (longer than three weeks). This period of three weeks has been based on the experimental findings of Salceda (102), who has demonstrated that rabbit corneal endothelium stored at 4°C for as long as 21 days could survive in a corneal button used for keratoplasty.

A statistical study was then undertaken in order to discover if a correlation exists between LDH % Residual after long-term storage and % Heart type isozyme of the

sample, at 4°C and at 23°C.

(c) Evaluation of Data and Further Tests on the LDH of Corneal Endothelium: A significant positive correlation between LDH %age residual after long-term storage at 4°C and % Heart type isozyme of the sample was established. The tissue extract of corneal endothelium of human and cat was then subjected to a calculation of the %age residual of the total LDH activity daily following storage at 4°C in order to verify the species variation in LDH isozyme patterns in the corneal endothelium obtained electrophoretically.

3. Results

(a) Relationship of LDH % Residual after Long-Term Storage to % Heart Type Isozymes: The % residual of total LDH in the sample stored longer than three weeks at 4°C has been calculated in 25 samples with various isozymic distributions. Fig. 17 (i) shows a regular increase in % residual related to % Heart type isozyme of LDH in the sample. The correlation coefficient of Pearson (44) between % Heart type isozyme (calculated in percent of LDH-1 + 3/4 LDH-2 + 1/2 LDH-3) and % residual activity of the total enzyme is + 0.81 ($p < 0.01$), which approaches unity and is highly significant. However, the variation is wide for the samples from human eyes, probably due to many uncontrollable factors,

while the conditions with cat eyes are easily controllable.

The slope of the estimated regression line (y = 1.05 x -10.3) is nearly 1, showing 1 : 1 proportionality between % residual activity of total LDH and heart type isozyme of the sample: The main loss of enzyme activity occurring with storage at 4°C is due to the inactivation of M-type isozyme; little inactivation of H-type is involved.

The % residual of total LDH in 24 samples stored at 23°C has also been plotted against % Heart type isozyme of the sample (Fig. 17. ii.). The correlation coefficient between % Heart type isozyme and % residual activity of the sample stored 23°C is only -0.06 ($p > 0.10$), which approaches zero and is not significant.

(b) Kinetics of LDH Inactivation in Corneal Endothelium: LDH inactivation kinetics at 4°C have been determined with tissue extracts of corneal endothelium from the human and cat.

It is seen in Fig. 18 that the main inactivation of LDH activity occurs in the first three days of storage at 4°C with apparently first-order kinetics (Fig. 18, Phase 1). The slope ($K = -\ln (\frac{A_R}{A_I})/T$) of the inactivation curve of this period is specific for the tissue extract of the corneal endothelium used: The inactivation rate constant as shown in Fig. 18 is approximately 2.7 times higher with human corneal endothelium than with cat corneal endo-

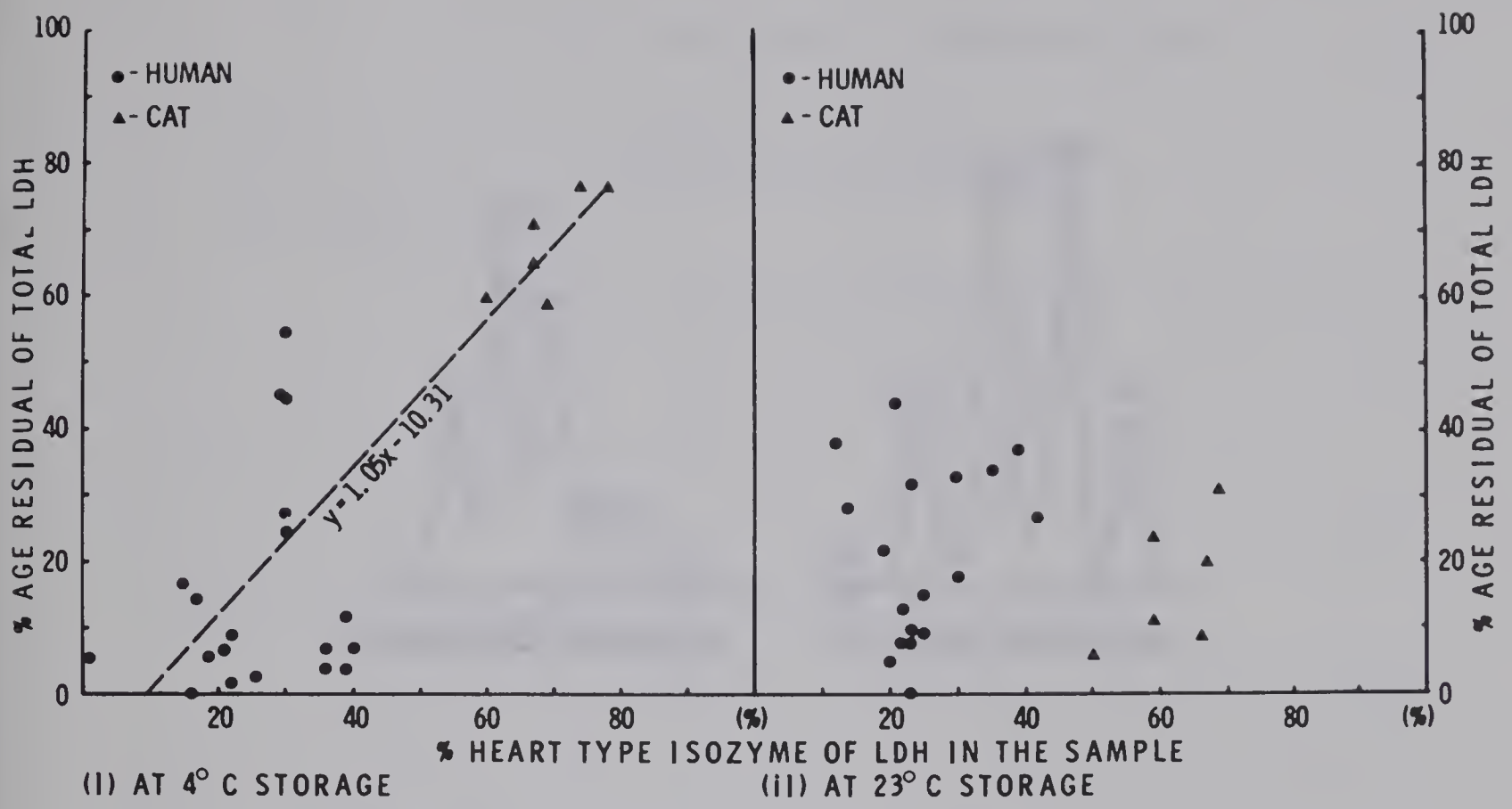
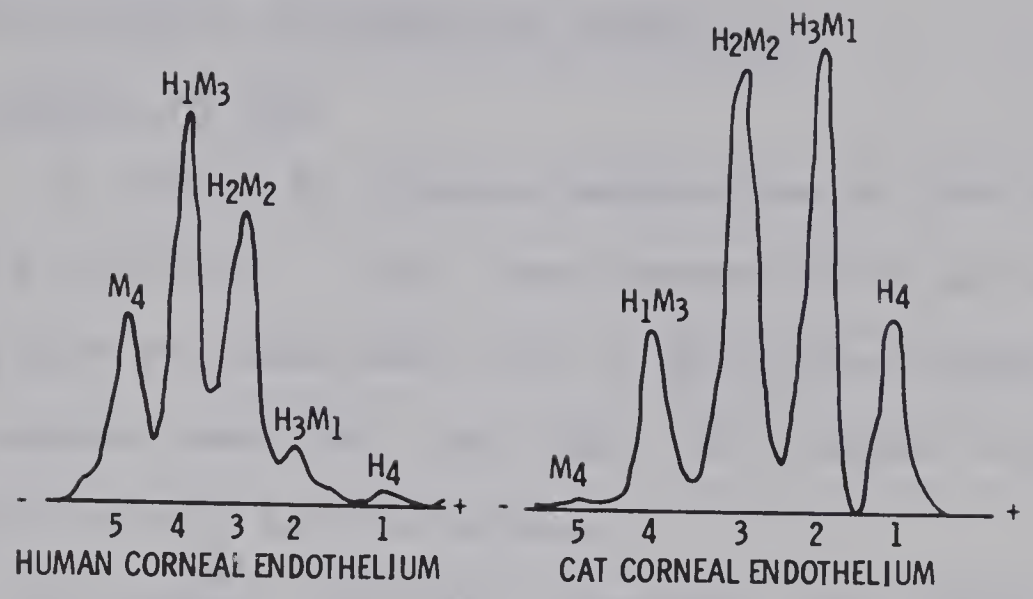


FIG. 1 - LDH % RESIDUAL AFTER LONG-TERM STORAGE VERSUS % HEART TYPE ISOZYME OF THE SAMPLE

Fig. 17. LDH % Residual after Long-Term Storage Versus % Heart Type Isozyme of the Sample

thelium.

The degree of inactivation after three days of storage (Fig. 18, Phase II) is negligible and nonspecific.



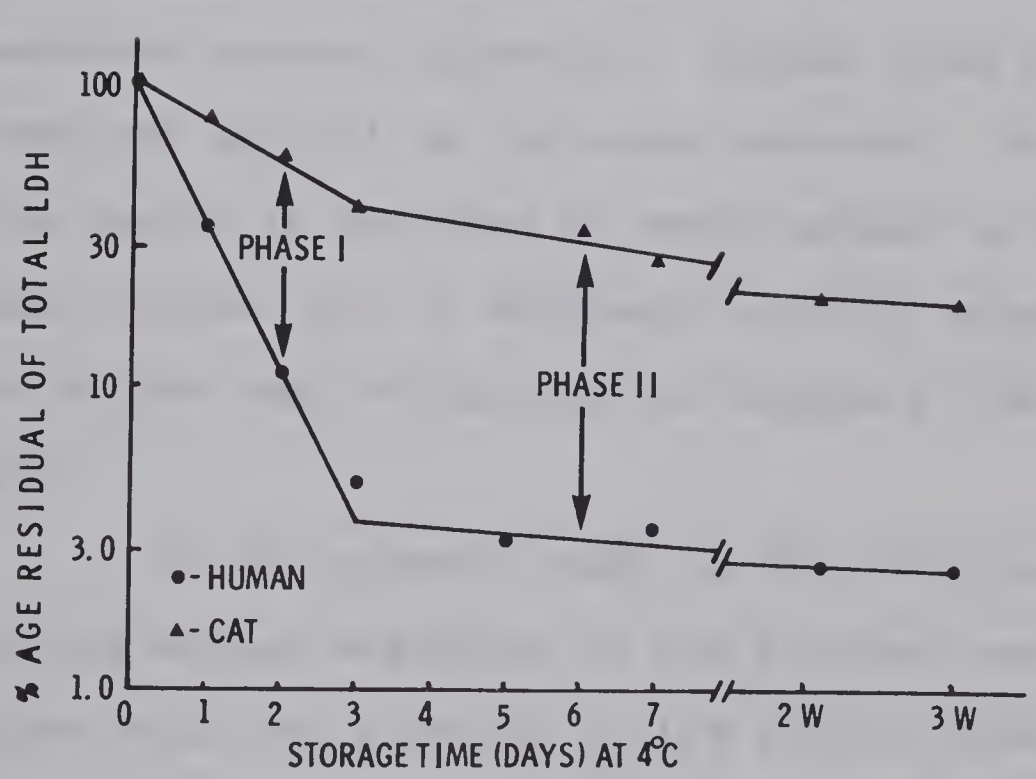


FIG. 2 - KINETICS OF LDH INACTIVATION IN THE COLD DETERMINED WITH CORNEAL ENDOTHELIUM

Fig. 18. Kinetics of LDH Inactivation in the Cold Determined with Corneal Endothelium

IV. DISCUSSION AND FURTHER INVESTIGATION

A. ON THE CORNEAL ENDOTHELIAL VIABILITY

1. The Nature of TRE

In regard to glucose metabolism in the cornea, it (2,62,63,106,107), has been assumed that an aerobic pathway plays an important role in producing energy to prevent corneal swelling, and that the glucose in aqueous supplies nutrients to the cornea.

As Smelser (106,107) observed, the oxygen supply from the limbal vasculature and via aqueous are inadequate to maintain corneal hydration. Oxygen from the atmosphere is required as well as the above sources. In other words, if the cornea is deprived of ready access to oxygen it becomes thicker due to decreased aerobic metabolic functions of the endothelium and the degree of TR should decrease.

In the present study on TRE using cat eyes, the rapid and marked breakdown of the glucose level in 24 hours together with the increase of L/P ratios could be explained by an increase in anaerobic glycolysis as shown in other tissues (43, 96) and by Reim and Turss (98) in bovine eyes.

Enucleation would of course disturb the oxygen supply to the endothelium, perhaps causing utilization of conventional anaerobic glycolysis to keep a steady state equilibrium of NAD^+/NADH. It has been shown (59) that all

of the oxygen disappears from the aqueous 25 minutes after blocking the blood supply to the eye.

The marked decline of the TRE in 24 hours to 33% of its initial value, closely parallels the marked fall in the glucose level in the aqueous in 24 hours to 1/3 of its initial level. This stresses the importance of glucose in the aqueous as an energy producing substrate possibly for the TRE particularly in view of the fact that the TRE is prevented if aqueous is removed before incubation.

Removal of the main source of energy production from the endothelium appears to produce a situation in which the swelling pressure of the corneal stroma, due perhaps to the Donnan effect or to a repulsion of charges within the mucopolysaccharide molecular complex, is greater than the counteracting forces, and results in further swelling during incubation.

The curve of the TRE after 24 hours however was approaching approximately 13% at prolonged storage time in the period of our experiment (138 hours), while the glucose in the aqueous dropped to almost 0 after 5 days. During this period of time the levels of lactate and pyruvate remained surprisingly constant giving a constant L/P ratio of about 100 (5 times that of fresh aqueous). The marked increase in L/P ratio which is characteristic of anaerobic glycolysis was not present, suggesting that the aerobic

oxidative metabolism in the corneal endothelium was still functioning, and that the high activity of anaerobic glycolysis was disturbed by the Pasteur effect; oxygen for this degree of aerobic activity could be supplied by diffusion through the whole cornea from the atmosphere as suggested by Takahashi and Fatt (115).

The positive TRE in this period of storage of the eye without aqueous but saline in the anterior chamber, suggests another factor influencing temperature reversal besides aqueous humor. Previous studies (38, 64) indicate that the cornea has a considerable reservoir which is utilized if the glucose supply is curtailed. The positive TRE without aqueous might therefore be explained on the basis of this reservoir. This would explain the constant L/P ratio during storage, and the fact that the TRE on both eyes with and without aqueous approached the same asymptote with increasing storage time.

The correlation of corneal transparency with substrate levels of an energy producing system in the endothelium (98) and the fact (Table I) that the TRE on eyes stored at room temperature decreases rapidly and approaches 0 in 2 days appears to rule out the role of a physico-chemical property in explaining the mechanism.

Glucose levels in the aqueous and corneal endothelium of eyes stored at room temperature drop to almost

0 within 10 hours (94,95), indicating that the suggested oxidative mechanism does not appear to function after storage at room temperature, certainly after 30 hours (98).

The second phase of metabolite fluctuation after 5 days with a further increase in lactate and a concomitant decrease in pyruvate seems to be caused by exhaustion of glucose.

2. LDH and Its Isozyme as a Measure of Corneal Endothelial Viability

If one compares Figs. 8 and 11 with Fig. 1, it can be seen that the inflection point of the curve for LDH and its 1-isozyme in their increase corresponds to that for the TRE in its decline i.e. at 24 hours of storage at 4°C. It is also obvious that TRE decreases markedly before this point and the LDH activity increases after this point. The fact that the inflection point of the change in the TRE and in the LDH level of aqueous both occur at 24 hours of storage is interesting since the TRE is assumed to be dependent upon a metabolically active endothelium (32).

These findings agree with the report by Jans (48), which shows a different slope of declining TRE prior to 24 hours storage from thereafter.

When one considers that relatively poor results are obtained with full thickness corneal grafts using corneas stored over 24 hours, the fact that the LDH level in

the aqueous of stored eyes increases quite markedly at 24 hours storage takes on some practical significance.

The increase of corneal endothelial cell specific enzymes in aqueous humor is attributed to the increase in permeability of the cell membrane, secondarily to cell damage. Therefore, the enzyme assay is not a direct test of endothelial cell viability. It does show indirectly, however, whether or not a cell is malfunctioning to the extent that its intracellular substance leaks into the aqueous humor.

The quantitative evaluation of LDH, and especially LDH-5 appears to be a simple method by which one can assess human donor material for corneal transplants without damaging the cornea.

3. Further Research: Mathematical Treatment of the Experimental Data

The results on TRE and on the endothelial viability have been interpreted only in an indicative qualitative sense.

TRE declined as storage time was increased, first rapidly up to 24 hours and then slowly approaching 13% at prolonged storage time up to 138 hours. TRE on 4 eyes stored at room temperature decreases rapidly and approaches 0 in 2 days. Based on this finding and the previous report (98) showing the correlation of corneal transparency with

substrate levels of an energy producing system in the endothelium, the role of a physico-chemical property if the stroma in explaining the effect has been qualitatively ruled out.

The problem then, arises whether the TR values of eyes stored at room temperature are in the confidence limit of TR of refrigerated eyes or significantly different from the latter. Fig. 1, which has been qualitatively treated as two hyperbolic curved lines can also be borne out by two separate straight lines instead of one curved line suggesting two different mechanisms involved in the TRE. The conflicting results of Jans (48) and Kirk (57) can then be explained by two independent processes. The fact that the inflection point of the change in the TRE and in the LDH level of aqueous both occur at 24 hours of storage time agree with the report by Jans (48), which also shows a different slope of TRE and number of stained cells at this point. The observation of Kirk (57) that the number of stained cells failed to increase with storage time of 50 hours up to 120 hours is consistent with the second part of the curved line of TRE after 24 hours (Result III.A.). Is the endothelial surface then, covered by a glycoprotein surface coat only in the prolonged storage time? Or, can the positive TRE in both eyes with aqueous and with aqueour replaced by saline, stored longer than 24 hours, be

explained by the physico-chemical characteristic of the stroma? It has been noted that the TRE remained fairly constant in spite of marked fluctuation in the level of metabolites (Result III. A.) after 5 days storage.

Quantitative evaluation is required in solving these questions using statistical analyses of the experimental data on TRE (Fig. 1) and on the LDH level (Fig. 7). The similarity of the curves to the growth curve of a cell population with a lag phase followed by an exponential phase also stimulates kinetic studies of TRE.

B. ON THE PHYSIOLOGICAL SIGNIFICANCE OF LDH AND ITS ISOZYMES IN THE CORNEA

1. Species Variation in LDH Isozyme Pattern of the Corneal Endothelium

In 1958, Markert and Möller (70) proposed a term "isozyme" to describe different molecular forms which exist with the same enzymatic specificity. The physiological significance of the isozyme is not fully realized. Isozyme may have a relation to control mechanisms in cellular metabolism, the so-called "metabolic control" according to Chance (8).

LDH isozyme has been studied in great detail and Cahn et al (6) have reported that LDH exists in five isozymes, each of which is a tetramer which is composed of two non-identical polypeptide chains, H and M, elaborated by

two different genes. If both the H and M genes are active in the same cell, then the primary gene products (Monomer units) recombine in some manner in groups of four, yielding five different molecular species (HHHH, HHHM, HHMM, HMMM, MMMM) all possessing LDH activity. According to this hypothesis, the subunits operate independently in respect to their substrate inhibition and rates of reaction with NAD analogs. Thus, the three intermediate LDH's differ quantitatively and qualitatively from the "parental" types much as genetic hybrids do, the enzymes being not the same but showing properties differing in a regular way from one extreme to the other. Based on the observation that there is a marked difference in the degree to which the activities of H_4(LDH-1) and M_4(LDH-5) are inhibited by pyruvate, Cahn et al (6) suggested that H-type is a catalyst geared for activity in an aerobic environment, whereas M-type functions in an anaerobic environment. The latter has been found in tissues showing a high rate of lactic acid accumulation.

In the cornea of the rabbit, Ueda (116) reported the LDH to be more dominant in H_4 and H_3M_1 as compared with that in the retina and in the iris and ciliary body: The epithelial-endothelial fraction of the rabbit cornea has been separated from the stroma by Graymore et al (28) and the possibility that the lactate formed by the stroma is utilized by the epithelium has been considered on the basis

of the difference in isozyme pattern between epithelial-endothelial fractions and stromal fractions.

The epithelial-endothelial fraction of the rabbit cornea showed a marked "H-" type isozyme, whereas that of the stromal fraction was most certainly an M-type.

The results obtained for cat corneas in the present work are in agreement with the observations for rabbit corneas by Graymore et al (28), but these authors did not distinguish between epithelium and endothelium. In the present experiment, the H-type isozymes are also found alone in the endothelium for cat cornea, but never for human cornea. The isozyme patterns of the human cornea are characterized by a predominance of LDH 3-5, not only in the endothelium and in the stroma, but also in the epithelium.

According to Stoner (112) and Hannon (30), the enzymes in the citric acid cycle and the related oxidative pathway, and also transaminase and Glucose-6-phosphatase, showed increased activity in the cold, so that the citric acid cycle intermediates would increase in amount.

The catalytic activity of the major molecular form in the cat corneal endothelial LDH, H-type, is, however, inhibited by low pyruvate concentration (6) and oxaloacetate at all concentrations (24), so that high quantities of pyruvate formed would inhibit the conversion to

lactate and divert it into oxidative sequence. Moreover, as Fritz (24) reported, the substrates in the Krebs cycle, as well as aspartic acid and glutamic acid, do not feed back and limit their own concentrations by activating H-type LDH as they do in M-type.

A highly resistant oxidative system shown by cat eyes during prolonged storage in the cold might be explained by the characteristic pattern of the endothelial LDH. The constant lactate, pyruvate and L/P ratio with rapidly decreasing glucose levels (Result III. A.) suggest that the corneal endothelium most probably utilizes lactate converted to pyruvate and the energy required is furnished mainly by the adenosine triphosphate produced as a result of the oxidation of citric acid cycle substrates.

On the other hand, LDH of the human corneal endothelium is an M-type. According to Fritz (24), LDH-5 is an allosteric protein: It is activated by seven citric acid cycle intermediates, as well as by aspartic and glutamic acids, which are directly converted to citric acid intermediates. Oxaloacetate, the remaining citric acid cycle substrate, is an analogue of pyruvate and is a competitive inhibitor of LDH-5 at concentrations greater than $7x10^{-4}$ M. However, this substrate activates LDH-5 at lower concentrations. This metabolic behaviour of LDH-5 gives a marked advantage of anaerobic glycolysis over the aerobic

respiratory pathway, since any activation of LDH would result in less pyruvate being available for conversion to acetyl-CoA and more NAD^+ being available to spark glycolysis through the reaction catalyzed by triose phosphate dehydrogenase. Thus, the human corneal endothelium would have a marked advantage over cat corneal endothelium when oxygen supply becomes limited as in the short term storage of enucleated eyes or under reduced oxygen supply imposed by lid closure during the night-sleep. The experimental fact that the epithelium of the bovine cornea is rich in lactic acid content (38) is in agreement with the high levels of the anoxic M-type isozyme, which favours lactic acid accumulation.

For the period of prolonged storage, of which endothelial metabolism has, however, been characterized by an oxidative system (Result III.A.), H-type, a major molecular form of LDH in cat corneal endothelium would give a marked advantage over M-type. For prolonged storage, it must be also considered that LDH_1 and LDH_5 have significantly different physico-chemical property regarding the stability of the enzyme molecule with temperature changes (Results III, A; C; D).

It is not surprising that the H-type is involved in the endothelial LDH of the cat, a nocturnal animal, and the M-type in the corneal endothelial LDH of the human, a

diurnal creature, if one dwells on the fact that lid closure during the night in sleep shuts off atmospheric oxygen and induces an increase of the corneal temperature. The M-type LDH of the human cornea, seemingly ideal for the diurnal rhythmic alteration of human life, has marked disadvantages in prolonged storage under standard eye bank conditions over H-type, which is most probably the characteristic feature of the endothelial LDH of the nocturnal animal.

2. Effect of Cold Storage on the Stability of LDH Related to Corneal Endothelial Viability

Plagemann et al (92) showed a complete inactivation of LDH-5 at 53°C for 6 min, whereas LDH-1 retains full catalytic activity for 40 min at the same temperature. Fritz (24) has described a difference in heat stability between LDH-1 and LDH-5. He also reported a desensitization of the enzyme LDH-5 in cold, but did not mention the effect of cold on LDH-1.

Studies on the effect of cold storage on the stability of LDH (Result III. D. a.) are also consistent with the effect of heat on LDH. The cold stability in terms of percentage residual increase simultaneously with an increase in percentage H-type isozyme in the sample. As the estimated regression line in Fig. 17 shows, a 90%-100% M-type isozyme (0%-10% H-type) will completely lose its catalytic activity with long-term storage at 4°C, while

100% H-type isozyme will be up to 95% stable. The loss of 5%-10% of the catalytic activity in H-type isozyme might be caused by a nonspecific inactivation, resulting from storage longer than three days, as shown by Phase II in Fig. 18.

It is seen in Phase I, Fig. 18 that the LDH of the human endothelial preparation is inactivated more rapidly and more markedly than in the cat. If one compares the slope of the curve, Phase I, as a rate constant on inactivation, the human endothelial preparation loses catalytic activity 2.7 times faster than in the cat preparation. Since the cold stability of the LDH can be directly compared with H-type (Result III. D.a.), this striking difference in the inactivation rate constant supports most convincingly the electrophoretic patterns obtained for the corneal endothelium of human and cat, which have shown M-type in the former and H-type in the latter with a ratio of "Heart" type isozyme, (Human corneal endothelium)/(Cat corneal endothelium) = 22.5/59.0 (Result III. C.) = 1/2.6.

It would appear, therefore, from these direct and indirect approaches of both electrophoresis and cold stability tests, that the human corneal endothelium has a typical "Muscle" pattern of LDH, while H-type being a major molecular form of LDH in cat corneal endothelium.

The highly resistant oxidative system in the cat

cornea and a positive TRE for the period of a prolonged storage at 4°C (Result III. A.) might be explained by the characteristics of the cat endothelial LDH.

Histological (104,108) and histochemical (50,99) studies have also pointed out the difference between rabbit and human of vulnerability of endothelium to damage in storage at 4°C. According to these reports, on the second storage day, human corneal endothelial cells exhibit irreversible morphological changes and many of them show absence of oxidative enzymes when examined histochemically (50,99).

The rabbit endothelium, on the other hand, degenerates slowly and after two weeks of storage, the majority of the cells are still viable by histochemical criteria (99).

The results reported here (Result III. D.) would help to explain this high resistancy of the corneal endothelium, H-type isozyme being stable in the cold.

M-type isozyme, a main constituent of human adult endothelium on the other hand, plays a major role in the inactivation of the LDH in storage at 4°C. A continuous increase in cold stability has been shown with the increase of M-type. Therefore, a correlation between this increase in cold stability from M- to H-type and the resistancy of the endothelial cells in storage at 4°C would be found,

although both phenomena may not be in a cause-effect relationship.

3. Further Research: Enzymes of the Cornea in Pathologic Conditions

Degenerative corneas may be more vulnerable to damage (74) in stress. Can we find any difference in "Metabolic control" in pathologic corneas?

The significance of LDH isozyme did not seem to be fully explained by mere difference in pyruvate inhibition, because pyruvate may be metabolized by mechanisms other than those entering the citric acid cycle and being converted to lactate. It is to be noted that regulatory mechanisms in glucose metabolism may be more complicated. In the present context, it (27) is important to note that when using a tetrazolium staining technique to identify the fractions, all five fractions are capable of transmitting electrons via either NAD or NADP. The activity of all five fractions is, however, far greater with the former coenzyme and there does not appear to be any difference of "activity ratio" between the fractions. Futterman and Kinoshita (25) nevertheless say in the LDH studies of cattle retina: "All these LDH components display activity with reduced NADP as well as reduced NAD and the relatively high ratio of NADPH to NADH activity compared to that reported for liver is in accord with the

postulation of a more significant role for LDH in the utilization of reduced NADP by ocular tissues." Moreover, the notably high proportion of glucose oxidized by pentose phosphate pathway (54) and the highest activity of LDH among enzymes participating in glucose metabolism in the corneal epothelium (60) lead to an assumption that LDH may play an important role in oxidizing NADP in the cornea. The high activity of this route would be also required by a considerable turnover of RNA, for which a high rate of the yield of pentose phosphate is necessary. Buñuel and Buñuel (5) have reported that the activity of G-6-PDH and 6-PGDH is increased in cells producing new connective tissue (protein synthesis) possibly in response to the increased requirement for ribose in RNA synthesis prior to collagen formation. Beaconsfield and Reading (2) have also observed that, under conditions of active protein synthesis, glucose oxidation by the pentose-phosphate pathway is stimulated.

In the preliminary work on enzymes in pathologic corneas (52) an enzyme abnormality, G-6-PDH in Keratoconus cornea has been presented. G-6-PDH dificiency has not been previously reported in this disease. It is suggested in this report that a G-6-PDH deficiency in the corneal epithelium in cases of Keratoconus, causes a lowering of reduced glutathione, and therefore lipid peroxidation of the lipid rich basement membrane, with resulting loss of

epithelial barrier function, loss of metabolites from the stroma and consequent stromal thinning, characteristic of Keratoconus. It is also speculated that the G-6-PDH deficiency may result in a decrease of pentose sugar for RNA synthesis, leading to improvement of stromal collagen.

The lipid peroxidation, which is an interesting aspect of lipid-rich membranes in many tissues under certain conditions (42), with resulting damage to structure and function. Keratoconus and cataract have been reported as constant findings in the turkey embryo deficient in vitamin E, an inhibitor of the peroxidative reaction.

O'Brien and Little (69,87,88) concluded that decomposition of lipid peroxides derived from unsaturated lipids in the presence of oxygen was due principally to reaction with the nucleophile glutathione by a mechanism catalyzed by the enzyme glutathione peroxidase. G-6-PDH deficient erythrocytes are unable to maintain their levels of reduced glutathione in the presence of low level, steady state concentrations of hydrogen peroxide (10).

The mode of reoxidation of NADPH produced in the pentose phosphate pathway has not been conclusively elucidated in the cornea. Kinoshita (53) suggested that pyruvate functions as an electron acceptor for NADPH on the basis of an inhibitory effect, on the pathway in the presence of

iodoacetate. However, the mode of the interaction between G-6-PDH and glutathione reductase in the corneal epithelium cannot be excluded.

Kinoshita et al (55) have found that, in certain circumstances, addition of oxidized glutathione stimulates the oxidation of glucose by the pentose phosphate pathway. This pathway is normally stimulated on incubation of glutathione-dificient red cells with methylene blue (93). According to Pirie (91), the autoxidation of ascorbic acid in the aqueous serves to oxidize glutathione in its surrounding tissues.

In Fuchs's dystrophy, there are microscopically fine pigmented granules and clumps of pigment scattered over the endothelium (11).

Halprin and Ohkawara (29) reported evidence that in human skin the activity of glutathione reductase and the concentration of glutathione are such that the oxidation of tyrosine to melanin by tyrosinase in inhibited. The possibility thus arises that the decrease of reduced glutathione level releases its inhibitory action on melanin formation.

Whether the same, so far entirely unknown, mechanism is ultimately involved in the Fuchs's dystrophy and special types of cataract, and in glutathione-deficient anemia is impossible to say at this moment. The similarity

between them, however, is that in glutathione deficient, nonspherocytic hemolytic disease, there Heinz bodies, a polymerized denatured hemoglobin molecules, result from oxidative injury and in Fuchs's dystrophy, Hassall-Henle bodies, intracytoplasmic hyaline droplets, most probably a structureless polymer of degenerated protein molecules, result. In cataract, there chemically an increase of the insoluble protein fraction and microscopically Morgagnian hyaline corpuscle are often noted.

The aqueous of the eyes and the systemic conditions of the patients should also be studied and correlated with the findings which will be obtained from the analyses of Fuchs's dystrophy, or of congenital cataractous lenses for the above enzymes and related substrates.

If the oxidative injury by any oxidant material in aqueous plays any role in developing Fuchs's dystrophy, its high coincidence with open angle glaucoma might be explained by postulating the same abiotrophy in the common endothelium lining the trabecular mesh work and the cornea.

It is unanimously agreed that women over 50 are much more frequently affected by Fuchs's dystrophy than men. A relationship between glutathione and estrone which can also be coupled to G-6-PDH by the transhydrogenation system has been reported (23).

The effect of such unknown factors and their pos-

sible synergistic effects in multiple step mechanisms make interpretation of results obtained clinically and histologically difficult.

Further work is required in the search for primary defects in the endothelium of Fuchs's dystrophy and in the possible pathways to induce Hassall-Henle bodies in order to clarify the interaction between the genetic and environmental factors in Fuchs's dystrophy. The possible source of oxidant material in the aqueous and its connection with the glutathione metabolism also suggests further investigation of the aqueous for substnaces which render the adjacent tissues capable of producing polymerized protein molecules, result from oxidative injury. Approaches directed to the regenerating system of $NADP^+$ in relation to the significance of LDH isozymes and to the lipid peroxidative reaction would be another interesting research area.

V. SUMMARY AND CONCLUSION

The study originally undertaken was made in order to develop a method that was simple, direct and accurate in measuring the endothelial viability of the cornea without damaging it for future use. It was decided to compare a method evolved in the present research utilizing enzyme analyses in the aqueous with the TRE following the methods of Hassard in 1964.

A. Studies on the TRE have been carried out on the corneas of enucleated cat eyes, with aqueous, and with aqueous replaced by saline. Several metabolites in the aqueous have been analyzed in the same eyes.

This work re-emphasizes the fact that the corneal TRE is a metabolic function of the cornea and suggests that glucose in the aqueous plays a major role. A secondary role is probably played by a substrate-reservoir in the cornea related to aerobic metabolism.

A surprisingly constant lactate and pyruvate and L/P ratio in the aqueous of refrigerated eyes with rapidly decreasing glucose levels during storage indicates a highly resistant oxidative system in the cat cornea.

The role of a physico-chemical effect in produc-

ing the TRE was ruled out by almost complete loss of the TRE in eyes stored at room temperature for two days.

B. The TRE was related to the enzyme levels in the aqueous. Of three enzymes tested, LDH and its isozymes are most significantly changed with increasing storage time.

A relationship between LDH levels in the aqueous and the TRE has been demonstrated, both sharing a marked change at 24 hours of storage. LDH and its isozymes are directly related to quantitative damage to the endothelium.

The release of LDH from endothelial cells has also been established by the concomitant decrease in enzyme activity in the endothelium.

Based on statistical studies using the aqueous of human eyes, it can be concluded that 50 mU/ml of LDH activity in the aqueous can be used as a borderline value to select the material under unknown environment.

Thus, analysis of aqueous for endothelial cell specific enzymes especially LDH and its isozymes provides a method for assessing corneal viability in eye-bank eyes without damaging the cornea.

C. The increased isozyme fraction in the aqueous after mechanical damage of the endothelium is LDH-5 in the human and LDH-1 in the cat. This species variation in LDH isozyme pattern agrees with that of the endothelial tissue extract itself.

The features of corneal endothelial metabolism under standard eye-bank conditions has been discussed in the light of the findings obtained, and an attempt has been made to explain the species difference in vulnerability of endothelial cells to damage during storage of the enucleated eye at 4°C.

D. The effect of the storage temperature on the stability of LDH-activity has been studied in tissue extracts of the ocular anterior segment, at 4°C and 23°C.

The kinetics of LDH inactivation upon exposure to cold have been investigated in corneal endothelial extracts of human and cat.

A significant positive correlation has been established between the cold stability index of LDH after long-term storage at 4°C and % Heart type isozyme in the sample.

A much higher inactivation rate constant has been found with the human corneal endothelium

than is the case with the cat, which is inversely proportional to the ratio of H-type isozyme in both samples.

This finding has a bearing on the species difference in endothelial cell viability in the cold and supports the isozyme patterns of LDH obtained electrophoretically for corneal endothelium.

E. The areas of further investigation are suggested in the search for primary enzyme defects in the corneal degenerative diseases in order to clarify the interaction between the genetic and environmental factors inducing these clinical and pathological features. The possible relation of the aqueous composition and the systemic condition of the patient with the pathogenesis of corneal defect has been discussed.

The physiological significance of LDH isozymes in relation to the corneal metabolism has been fully discussed utilizing the findings reported here. Further work should then involve approaches directed to the regenerating system of $NADP^+$ for pentose phosphate pathway.

The mechanisms of TRE have been questioned also whether the effect is one continuous process or two separate ones. Quantitative evaluation using mathematical analysis of the experimental data col-

lected here may answer the question perhaps aided by kinetic studies of the TRE.

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